REVIEW ARTICLE

Mechanisms balancing skeletal matrix synthesis and degradation

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Bone is regulated by evolutionarily conserved signals that balance continuous differentiation of bone matrix-producing cells against apoptosis and matrix removal. This is continued from embryogenesis, where the skeleton differentiates as a solid mass and is shaped into separate bones by cell death and proteolysis. The two major tissues of the skeleton are avascular cartilage, with an extracellular matrix based on type II collagen and hydrophilic proteoglycans, and bone, a stronger and lighter material based on oriented type I collagen and hydroxyapatite. Both differentiate from the same mesenchymal stem cells. This differentiation is regulated by a family of related signals centred on bone morphogenic proteins. Fibroblast growth factors, Indian hedgehog and parathyroid hormone-related protein are important in determining the type of matrix and the relation of skeletal and non-skeletal structures. Removal of mineralized matrix involves apoptosis of matrix cells and differentiation of acid-secreting

cells (osteoclasts) from macrophage precursors. Key regulators of matrix removal are signals in the tumour-necrosis-factor family. Osteoclasts dissolve bone by isolating a region of the matrix and secreting HCl and proteinases at that site. Successive cycles of removal and replacement allow growth, repair and remodelling. The signals for bone turnover are predominantly cell-membrane-associated, allowing very specific spatial regulation. In addition to its support function, bone is a reservoir of Ca^{2+} , PO_4^{3-} and OH^- . Secondary modulation of mineral secretion and bone degradation are mediated by humoral signals, including parathyroid hormone and vitamin D, as well as the cytokines that also regulate the underlying cell differentiation.

Key words: apoptosis, macrophage, mesenchymal stem cell, osteoblast, osteoclast.

INTRODUCTION

The skeletal matrix in air-breathing vertebrates, including humans, undergoes continual site-specific degradation that is balanced by new bone synthesis. The resulting turnover allows shape to be modified during growth, and damaged bone is replaced with new bone rather than by scar tissue. Additionally, mineral is deposited and removed for calcium and acid–base homeostasis. Because of this metabolic activity, skeletal turnover occurs at a rate exceeding that needed for growth or repair, usually by a wide margin. Central elements of most of the biochemical pathways for skeletal formation and degradation have been defined, including key mediators of the cell-differentiation pathways for chondroblasts, osteoblasts and osteoclasts. Bone formation and degradation are mediated by differentiation of skeletal cells from pluripotent precursor cells in a manner closely related to differentiation during skeletogenesis. Key features of embryonic bone development will be discussed below, showing the relationship to bone turnover and remodelling. Skeletogenesis is driven by cellular and hormonal mechanisms that, with minor modifications, also control bone turnover, reflecting the fundamental linkage of skeletogenesis and bone metabolism. Programmed cell death, apoptosis, is the central process that controls bone shape. Apoptosis divides the skeleton

into individual bones. In later development, programmed cell death permits replacement of cartilage by bone, and, in mature bone, turnover depends on apoptosis of osteoblasts and osteoclasts. In this review, we will describe the major biochemical elements of bone metabolism, with emphasis on physiological mechanisms that control cell differentiation and programmed cell death.

COMPONENTS OF VERTEBRATE BONE REFLECT EVOLUTIONARY MILESTONES

Vertebrate specialization is accompanied by increasing skeletal complexity. The primitive vertebrate structure is characterized by a solid axial skeleton; its most basic form is the notochord. In most classes of vertebrates the axial skeleton is divided to form individual skeletal elements, and appendicular and dermal (limb and skull) elements are then added. Because the skeletal primordia condense as continuous columns of matrix, apoptosis and proteolysis are primary to this sequence. The mesenchyme differentiates into cartilage. Mesenchymal stem cells are retained at the periphery of cartilages, and produce specialized tissues that augment or replace cartilage.

Abbreviations used: PTH, parathyroid hormone; PTH-rp, PTH-related protein; TGF- β , transforming growth factor- β ; BMP, bone morphogenetic protein; GDF, growth/differentiation factor; NF- κ B, nuclear factor- κ B; IL, interleukin; TNF, tumour necrosis factor; FGF(R), fibroblast growth factor (receptor); IHH, Indian hedgehog; RANK, receptor activator of NF- κ B; RANKL, RANK ligand; CSF-1, colony-stimulating factor 1; TRAF, TNF-receptor-associated factor.

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Chondrocytes

Cartilage is synthesized by individual cells, chondrocytes, that secrete, and surround themselves with, an avascular extracellular matrix. Chondrocytes differentiate from mesenchymal stem cells, and are capable of cell division within the cartilaginous matrix for growth or replacement. Chondrocytes produce randomly oriented type II collagen, which provides tensile strength, and hydrophilic proteoglycans. Aggrecan, the major component, is a chondroitin sulphate proteoglycan that binds to hyaluronan and provides a firm base for the elastic matrix. This hyaline cartilage is the terminal skeleton in chondrichthyes (cartilaginous fishes, e.g. sharks and rays) and, in subsequent classes of vertebrates, the skeleton is mainly formed initially in hyaline cartilage. Several other proteoglycans are also present in cartilage that have specialized functions often related to formation of complexes and cellular attachments [1].

Calcium homeostasis and the osteoclast

In bony fishes (osteichthyes) the skeleton is largely mineralized, increasing resistance to compression, but the mineralized matrix is acellular [2]. The static acellular matrix of the bony fishes is retained only in parts of the teeth of air-breathing vertebrates. A key advance, which appears in teleost fishes that inhabit both salt- and fresh-water, is the development of osteoclasts that degrade mineralized matrix [3]. In addition to permitting skeletal remodelling, this allows the mineral to be used for homeostasis of circulating calcium. Osteoclasts are multinucleated cells generated by the fusion of pluripotent monocytes from blood. The resulting syncytial cells are specialized for acid secretion at the bone surface to dissolve mineral, and for enzymic degradation of the organic matrix uncovered by removal of the mineral. Osteoclast formation is controlled by parathyroid hormone (PTH). This soluble protein derives from, and shares receptors with, the ancestral cell-membrane signal PTH-related protein (PTH-rp). PTH-rp is a key differentiation signal in several mesenchymal cell types and is crucial in cartilage/bone differentiation decisions in the terrestrial vertebrates (see below). PTH is secreted by branchial (gill) organs, the parathyroid glands, which have membrane receptors that respond to the activity of ionized calcium. When $[Ca^{2+}]_a$ declines, PTH is secreted and osteoclasts are formed, which dissolve bone mineral, thus restoring calcium activity.

Modifications for life on land

Terrestrial vertebrates form the skeleton mainly as miniature models in cartilage, and then replace the models with a new type of skeleton, cellular bone, which is stronger and lighter than the acellular bone of osteichthyes. Mass is reduced by removing most of the matrix, while key portions are replaced by the stronger bone to compensate. To create this new skeleton, individual cartilages are first subdivided. Chondrocytes mineralize part of the matrix while degrading the remainder with metalloproteinases to initiate the transformation, and are removed by apoptosis. This occurs in specific locations within the cartilaginous bones to create mineralizing 'centres' that replace the bulk of the cartilage. Plates of hyaline cartilage are retained that transect the long axes of long bones, typically near the ends of long bones. These 'growth plates' allow for lengthening of the bones by division of chondrocytes within their matrix. Osteoclasts attack the mineralized cartilage surfaces left by apoptotic chondrocytes. Since osteoclasts differentiate from circulating cells, vascular invasion of the bodies of the primordial cartilaginous bones is required. The mineralized matrix and spaces left by

apoptotic chondrocytes create surfaces upon which a new type of mineralized cellular matrix differentiates. This cellular matrix forms a cortex of solid bone at the periphery of the old skeleton, that is connected by struts where reinforcement is required. This bone is stronger, lighter, and has a much larger surface area than acellular bone.

Osteoblasts

Osteoblasts differentiate from the mesenchymal stem cells that also produce chondrocytes. However, the osteoblasts of terrestrial vertebrates incorporate key advances. The new bone matrix is based on cross-linked type I collagen ($\approx 10\%$ of bone mass) in oriented laminae alternately parallel and orthogonal to the major axis of flexion of the bone; into this dense collagen, mineral with stoichiometry similar to hydroxyapatite $[4 Ca^{2+} + 2 PO_4^{3-} + 2 OH^-]$ (subject to variable ion substitution, especially in surface layers) is generated. Bone collagen is remarkable for being more highly cross-linked at lysine and hydroxylysine residues than other tissues, although the formation of cross-links is not known to have any features specific to bone. The resulting matrix, a reinforced composite of oriented cross-linked collagen and ceramic-like mineral, is extremely strong. This bone is organized in multicellular units of osteoblasts connected by gap junctions, called osteons; these units facilitate the organization of the composite structure by forming units typically 6–7 cell-layers deep and 50–100 μm in average surface diameter. Each osteon retains collagen strand orientation, and the individual cells remain alive and interconnected during the life of the functional bone unit. After initial osteon formation, a process of sequential degradation and formation results from cycles of regulated osteoclast and osteoblast cell differentiation and apoptosis. By regulating where bone is removed or deposited, the processes of growth, repair and mineral homeostasis are governed.

REGULATORY SIGNALS AND SKELETAL DIFFERENTIATION

Skeletal development in vertebrates is controlled by homeotic (*hox*) genes, which encode conserved DNA-binding transcription factors that regulate genes in early differentiation. *Hox* genes are homologues of an ancestral gene system that controls body patterning in all metazoans, including invertebrates. One function of *hox* genes is to produce repeating units of identical cells, in segments. *Hox* genes are expressed in the order of their linear arrangement on chromosomes, and in vertebrates and humans there are four sets: A, B, C and D on chromosomes 7, 17, 12 and 2 respectively [4]. The boundaries of expression of these regulatory genes allow the segmentation of the primordial skeleton [5] via intercellular signals expressed at these sites. *Hox* gene expression is positively regulated at segmentation junctions of the embryo. This expression trails from segment boundaries, and tends to overlap on the next *Hox* domain (reviewed in [6]). *Hox* genes are also critical in body patterning beyond segmentation, including induction of limb buds. Because of their fundamental roles, loss of *Hox* genes causes serious lesions, and only deletions of terminal *hox* genes are consistent with survival, often with serious developmental defects. Hemizygous states missing entire terminal *hox* clusters allow development to term, although with complex developmental defects [4]. For more comprehensive discussions of patterning and differentiation, see [7].

Transforming growth factor- β (TGF- β)

Segmentation, growth and condensation driven by homeotic genes defines broad domains in the embryo. The mechanisms of

Figure 1 TGF- β superfamily genes are critical to defining the shape of individual skeletal elements

In these photographs, hybridization of probes for type II collagen (A and C) and GDF5 (B and D) is shown in hind-limbs of mouse embryos. At day 12.5 (top pair) the future bones of the digits are still part of a single cartilaginous precursor. By day 14.5 (bottom pair) the cartilage precursor is segmenting, with future joint areas recognizable as regions of decreased collagen II expression, and increased cell death. GDF5 is expressed specifically in the regions of joint formation, and is required for joint formation to occur. TGF- β family members, particularly of the BMP and GDF families, have very complex promoters that define where specific structures form. They can stimulate cartilage and bone formation, but also can stimulate cell death and other responses depending on developmental stage and context. A balance of signals, such as TGF- β family proteins and antagonists (noggin) and FGFs, determines where skeletal elements grow and differentiate and, as importantly, where apoptosis allows division of separate bones and formation of joint spaces. Reproduced from [15] with permission. © (1999) Academic Press.

these processes include cell division, differentiation and programmed cell death (apoptosis). In skeletal cells, the TGF- β superfamily of genes, which encode a large number of dimeric extracellular signalling molecules, are primarily responsible for the control of these mechanisms. This superfamily includes bone morphogenetic proteins (BMPs) and growth/differentiation factors (GDFs) [8]. TGF- β superfamily genes are expressed throughout life. Signalling for the TGF- β superfamily involves serine/threonine receptor kinases of two types, I and II. Type I is the signalling receptor, but it is inactive unless associated with a type II, constitutively active, kinase. These receptors can form homo- or hetero-dimers when they bind their dimeric ligands. The total number of receptor proteins is less than the size of the BMP family, so that BMPs produce overlapping effects, depending on cellular receptor and ligand protein expression. Other characteristics of the BMP family include the secretion of inactive dimers that are activated by matrix metalloproteinases, and that some BMPs are monomeric proteins that act as suppressors.

BMP regulation of transcription

Intracellular signalling by BMP superfamily proteins is divisible into two subclasses, depending on the DNA transcription cofactors (smads) that they activate: AR-smads, smads 1, 5 and 8; or BR-smads, smads 2 and 3 [9]. TGF- β and activin signals regulate AR-smads. They are responsible for growth suppression and differentiation in many tissues, but in fibroblasts and osteoblasts are growth factors. The BMP and GDF proteins that are important in skeletal patterning and differentiation of skeletal cells activate BR-smads [10]. *Smad* genes were identified as homologues of the *Drosophila mad* (mothers against decapentaplegic) and *Caenorhabditis elegans sma* genes [11]. Smads participate in the activation of a variety of transcription factors. Their activity is regulated by kinases, including the BMP type I and II receptors. Smad3, a BR-smad, is a cofactor for the vitamin D receptor and nuclear factor- κ B (NF- κ B), which are both important in bone cell differentiation [12,13]. A variety of proteins that are not phosphorylated by BMP receptors modulate smad activity, including Hox transcription factors. [14].

BMP control of cellular and tissue differentiation

BMP genes have complex promoters that express the genes in highly specific patterns [15] (Figure 1). At those locations, BMP-induced differentiation patterns result in cells of two principal phenotypes, chondroblasts and osteoblasts. The paradigm of BMP expression patterning with common intracellular signalling pathways is useful in understanding skeletal differentiation. The resulting osteoblasts are generally indistinguishable, regardless of the skeletal location. There are identifiable subclasses of chondrocytes. In repair tissues at special locations, including the joints of the vertebral column, and in the developing skull, these subtypes produce matrix with differing proteoglycan, elastin and collagen contents. The structural distinctions of these molecules have been incompletely described. However, the uniformity of the hyaline cartilage and osteoblasts throughout the skeleton is striking. Thus the multiplicity of BMPs through homeotic genes produce a small number of cellular phenotypes. This is recapitulated in mature terrestrial vertebrates, where functional mesenchymal stem cells circulate and may differentiate under regional control [16]. BMP-2 and BMP-4 are also expressed in mature bone, and probably function in skeletal remodelling.

Regulation of BMP function and programmed cell death

Soluble BMP-binding proteins, 'decoy receptors', are important regulators of BMP function. They trigger cytokine-withdrawal apoptosis during skeletal differentiation. These soluble receptor-like proteins sequester BMPs. Soluble receptors for several TGF- β superfamily proteins are known. One of these, noggin, shows regulated expression in skeletal mesenchymal cells [17]. In this way, noggin controls apoptosis and regulates chondrogenesis in the developing skeleton [18]. Transgenic mice lacking noggin have a fused appendicular skeleton, and die perinatally. In humans, heterozygous mutations in noggin are found in families with joint fusions [19]. These defects result because the defective noggin does not promote programmed cell death, forming joint spaces or subdividing the limb buds to produce parallel bones and digits. Gremlin, a less well characterized BMP-binding protein [20], is also required in skeletal patterning. Binding proteins also regulate other cytokines that are important in bone differentiation, including interleukin (IL)-1 and tumour necrosis factor (TNF) family proteins, as described below.

Modification of patterning by fibroblast growth factors (FGFs)

Primitive segmentation-related genes establish the positional development of the skeleton, but this is modulated by key secondary structures, including apical ectodermal ridges and associated cytokines, which include FGFs and sonic hedgehog [20]. Similarly, the skull, which develops in proximity to the ectoderm, is influenced by FGF-regulated pathways discovered by examination of genetic anomalies [21]. The proliferation-promoting FGFs function in early embryonic differentiation, along with TGF- β family genes, to co-ordinate patterning and cell differentiation. However, what is best understood about FGFs and skeletal regulation is that these genes are essential to development at sites of close interaction between mesenchymal and epithelial cells [22]. These occur in the formation of the skull and in the limb buds. FGFs are heparin-binding proteins that occur as heterodimers and oligomers with heparin, so that interaction with the membrane FGF receptors (FGFRs) produces receptor dimerization and tyrosine kinase activation. Four FGFRs have been identified; each has three immunoglobulin-like extracellular domains. In contrast, nineteen FGFs are known in vertebrates, which are synthesized in tissue-specific patterns [23]. As with TGF- β proteins, many FGFs interact with a few receptors. This overlapping interaction pattern emphasizes that the sum of local FGF molecules can drive limb outgrowth and other segmental developmental features [24].

FGFR action in the skeleton

Focal FGFR mutations and splicing defects that impair tyrosine kinase activity cause significant developmental anomalies. These include craniosynostosis (fusion of skull plates) and syndactyly (fusion of digits). The focusing of many FGF ligands in overlapping patterns on FGFRs produces predictable results in transgenic animals (reviewed in [25]). FGFR1 and FGFR2 are both essential for early embryonic development. In particular, defects in FGFR2 are associated with defective limb formation. In contrast, FGF-null mutations, such as FGF8 and FGF10, may create defects limited to later development, and may be less severe [26].

Other developmental regulators

Sonic hedgehog and Wnt function in concert with TGF- β family signals and FGF at skeletal boundaries that have common features at many sites, such as chondrocytes adjacent to joint spaces or cartilage–bone interfaces. Sonic hedgehog is a secreted ligand that activates Gli nuclear-transcription factors necessary for mesenchymal patterning, including segmentation and tissue separation. Wnt is crucial to both embryonic patterning and stem cell regulation in tissue replacement of mature animals [27]. Wnt signals interact with TGF- β at the level of smads to regulate local differentiation [28].

REPLACEMENT OF CARTILAGE BY BONE

The conversion of cartilage into cellular bone requires several processes. Chondrocytes dissolve part of their matrix, mineralize the remainder, and die. Growth of vascular tissue into bone delivers osteoclast and mesenchymal precursors into the bone. The mineralized matrix is removed by osteoclasts. The degraded matrix is replaced by cellular bone formed by osteoblasts acting in organized units. Ultimately, the new bone matrix production depends upon changing the differentiation of pluripotent stem cells from chondrocytes to osteoblasts. Several key molecular mechanisms that regulate these events are known, although substantial gaps in understanding remain.

Chondrocyte differentiation

Chondrocyte differentiation is closely integrated with overall skeletal development. Chondrocytes and osteoblasts make many shared proteins, and during embryological development common factors are involved in the regulation of both cell types. The nuclear transcription factor Sox9 is required for cartilage differentiation; its absence causes a lethal skeletal malformation, campomelic dysplasia [29]. The bone-specific transcription factor *cbfa1* is required for normal cartilage differentiation [30]. During skeletal development, *cbfa1* expression is controlled by *Hoxa2* and *Sox9* [5]. Chondrocytes are dependent upon continuing cytokine stimuli, and undergo apoptosis when they are withdrawn [31].

Mineralization of cartilage in transition

Mineral deposition occurs between columns of hypertrophic chondrocytes, by active secretion that requires 1,25-dihydroxy-vitamin D. Angiogenesis, stimulated by membrane metalloproteinase 1 [32], is obligatory for the introduction of osteoclast precursors. Osteoclasts are responsible for mineral removal, and only mineralized cartilage is degraded by these cells. These cells are indistinguishable from the osteoclasts of cellular bone. Supported by vascularization, which is also necessary for undifferentiated mesenchymal stem cells to reach the interior of cartilages, osteoblasts differentiate on the plates of mineralized cartilage between the columns of hypertrophic chondrocytes. These form the 'primary spongiosa', on to which trabecular bone is deposited (Figure 2).

Chondrocyte decline

The mineralization of cartilage begins at sites in the diaphysis (shaft) and epiphyses (ends) of bones, ultimately reducing the cartilage until it is limited to growth plates and joint surfaces. Embryonic cartilage at these sites is converted into bone, beginning in the late embryonic period (Figure 2A). The initial stage of cartilage conversion into bone involves increasing the size of chondrocytes (hypertrophic chondrocytes), which secrete metalloproteinases and deposit mineral in matrix. The matrix metalloproteinases (MMPs), including MMP2, MMP9 and MMP13, participate in resorption of the non-mineralized matrix [33]. Metalloproteinase activity connects hypertrophic chondrocytes in discrete columns. The hypertrophic chondrocytes die, and the mineralized matrix between columns left by this process is the template of trabecular bone. The process of chondrocyte hypertrophy ends in cell death, characterized by nuclear condensation and cytolysis. Cellular signals associated with apoptosis occur in hypertrophic chondrocytes [31,34].

OSTEOBLASTS IN TERRESTRIAL VERTEBRATES

Pluripotent mesenchymal cells produce chondrocytes, fibroblasts, myocytes, adipocytes and osteoblasts [35]. In terrestrial vertebrates, osteoblasts are connected by gap junctions composed largely of connexin 43. These electrically connected groups of osteoblasts, the activity of which can be precisely synchronized [36]. These units, osteons, promote co-ordinated synthesis of sheets of bone. Osteoblast-competent stem cells occur throughout the body, but differentiation is limited to the skeleton, where appropriate cytokines are expressed. Exceptions occur in inherited disorders, such as fibrodysplasia ossificans progressiva, in which BMP-4 or Fos is expressed ectopically [37].

Figure 2 Cell differentiation in bone

(A) Differentiation between cartilage and bone. A schematic view is shown adjacent to a 1×2 mm section from a human growth plate (haematoxylin and eosin; cartilage proteoglycans are blue and bone collagen is red). Once a cell differentiates into cartilage or bone, it cannot revert lineage. Ongoing differentiation in bone requires mesenchymal stem cells, which differentiate into several cell types. In the transition between cartilage and bone, feedback between Indian hedgehog and PTH-rp is critical, as are differences in FGFRs and BMP expression. Many other factors are required for normal differentiation, including vitamin D. The formation of hypertrophic chondrocytes in columns divides the developing bone into apoptotic regions, which become inter-trabecular spaces, and the mineralized columns, which are the scaffold for osteoblast bone deposition as the remaining mineralized cartilage is degraded. (B) Continuing differentiation of osteoclasts from marrow monocytes and osteoblasts from pluripotent mesenchymal stem cells. Osteoclasts differentiate under the influence of pro-apoptotic signals including RANK-ligand (RANKL) or $\text{TNF}\alpha$, and CSF-1. Many accessory cytokines are important to the efficiency or regulation of the process, although intermediate stages of development are not well characterized. Development of osteoblasts is dependent on BMPs and PTH-rp. As in osteoclast development, other factors including vitamin D are necessary, and a host of cytokines act as co-regulators.

Appearance of osteoblasts

The shift of mesenchymal stem cells from chondrocyte to osteoblast differentiation involves FGF, BMP, PTH-rp and Indian hedgehog (IHH) [38]. Because these proteins are expressed in cartilage, considerable effort has been expended to determine how the switch from cartilage to bone occurs. PTH-rp and IHH are particularly important in determining the terminal cell type. IHH is a conserved *Drosophila* segmentation gene downstream of the homeotic signals. It is required for chondrocytes to progress into the 'hypertrophic' process of mineralization, proteinase secretion and apoptosis. IHH interacts with many other genes that regulate cartilage and bone cell proliferation, including inducing PTH-rp. cAMP is the primary intracellular signal produced by PTH-rp [39]. IHH induces expression of PTH-rp, which inhibits IHH in a negative feedback loop that maintains cartilage in its untransformed state [40], in which FGF3 expression is an important functional element. When PTH-rp is withdrawn, cartilage senescence, mineralization and osteoblast replacement are initiated. Subsequently, FGFR1 is expressed in hypertrophic cartilage cells as they mineralize the matrix and secrete metalloproteinases [41].

Mineralization

Mineral deposition is the least well understood aspect of skeletal metabolism. Although there is a large body of literature, a well-defined biochemical transport pathway remains elusive. It is clear that mineralization requires active cellular transport, and the best evidence points to a mechanism on the basis of phosphate secretion, probably incorporating alkalization of the matrix, as well as an increase in the concentration of phosphate locally [42,43].

Accessory matrix proteins

Deposition of hydroxyapatite-based mineral requires an alkaline phosphatase; in its absence (hypophosphatasia), pyrophosphate accumulates. Thus pyrophosphate is an intermediate in mineralization, although the complex phosphates that yield pyrophosphate for matrix mineralization are not identified with certainty [44]. Several proteins besides alkaline phosphatase, including osteopontin and osteocalcin, are produced by osteoblasts and are important in maturation of normal mineral matrix. Osteocalcin is a calcium-binding protein that contains γ -carboxyglutamic acid [45]. Osteocalcin is synthesized in a significant quantity only in the osteoblast.

Osteoblast-specific promoters

Because osteocalcin is limited to the osteoblast, analysis of its promoter has provided important information about terminal osteoblast differentiation. This is controlled largely by the *cbfa1* transcription factor, also called *osf2* [7]. The *cbfa1*-binding site, in tandem with less-well-characterized proximal binding sites in the osteocalcin promoter, is specific for the osteoblast [7,46]; defects in *cbfa1* are one cause of osteopenia. Transcriptional control by PTH and vitamin D is also well characterized, and these are important elements regulating terminal osteoblastic differentiation and activity, about which numerous studies exist. PTH stimulates bone synthesis (but not mineral deposition) by osteoblasts, and also mineral removal by osteoclasts. As a consequence, PTH causes an increase in the amount of non-mineralized bone (osteoid).

Osteoblast regulation of matrix removal

Osteoblasts produce important regulatory proteins, including the TNF family protein RANKL [receptor activator of NF- κ B

(RANK) ligand], which increases osteoclast number. This regulatory system is depicted in Figure 2(B). Production of RANKL by osteoblasts is stimulated by PTH, vitamin D, IL-1 and several other cytokines including IL-6, which signal through the gp130 receptor subunit [47]. Complex intracellular mechanisms balance PTH and other control mechanisms, such as stretch-related responses, in osteoblasts [48]. The terminal fate of the osteoblast is apoptosis, driven by mechanisms including TNF receptors, as discussed below.

CALCIUM-REGULATING HORMONES

PTH-rp, PTH and vitamin D regulate rates of differentiation and mineral transport at multiple points, and are key co-ordinators of skeletal development and metabolism. PTH-rp is an important extracellular signal that regulates osteoblast and chondrocyte proliferation. The circulating hormone PTH activates the same receptors as PTH-rp, and appears to have arisen by gene duplication, with PTH-rp probably being closer to the ancestral function. Two PTH receptors have been identified [49]. PTH receptor type 2 is expressed in neural endocrine and in reproductive tissues [50]. Type 1 is involved in skeletal and related metabolism, and is found in osteoblasts, kidney and other mesenchymal cells. The parathyroid gland differentiates under the control of the *gcm2* transcription factor, which is a homologue of a conserved transcription factor involved in neural and thymus differentiation (*gcm1*). Curiously, when *gcm2* is ablated in mice, parathyroid glands do not form, but PTH is expressed in the thymus [51], suggesting that *gcm2* and *gcm1* are products of gene duplication that retain a functional overlap.

PTH and calcium in skeletal metabolism

A key physiological response to PTH is an increase in serum Ca^{2+} . PTH dramatically increases osteoclastic differentiation and activity. However, the osteoclast has little, if any, direct response to PTH. PTH receptors on mesenchymal cells, particularly the osteoblast, regulate secondary signals that cause osteoclast formation. PTH also directly increases bone synthesis, but not mineralization. This relationship, which is reflected in effects of PTH on expression of many bone proteins, is important in preserving skeletal matrix volume when bone is degraded to maintain serum Ca^{2+} . The local signals that regulate bone degradation by osteoclasts are discussed in the next section, but it should be noted that PTH-responsive proteins include osteoprotegerin, which limits activity of selected TNF family proteins including RANKL [52]. PTH maintains osteoblast survival *in vitro* [53], and C-terminal fragments of PTH are implicated in osteoblast differentiation [54].

Vitamin D

1,25-Dihydroxyvitamin D is a steroid hormone required for mineralization of cartilage and bone matrix, and promotes osteoclast differentiation. Vitamin D is required for mineralization and degradation of bone, but only at very low vitamin D concentrations is skeletal development arrested [55]. Under physiological conditions, a renal 25-hydroxylase regulates the activity of vitamin D, which serves largely to balance renal, intestinal and skeletal calcium and phosphorus transport.

The vitamin D receptor is a nuclear transcription factor. Binding of 1,25-dihydroxyvitamin D results in a conformational change in the vitamin D receptor that enhances the interaction of retinoid D receptor with the retinoid X receptor. Vitamin D response elements in genes are recognized by an active retinoid D receptor–retinoid X receptor heterodimer. Activation of the

vitamin D receptor has variable effects, depending on cellular differentiation and on interactions with other transcription factors. TGF- β increases cell proliferation and expression of the vitamin D receptor in osteoblasts, although in the presence of vitamin D, cellular maturation with expression of important matrix proteins, including type I collagen and alkaline phosphatase, occurs [56]. In contrast, effects of 1,25-dihydroxyvitamin D alone include changes in expression of many important proteins, often in contrasting directions, including negative regulation of type I collagen [57] and *cbfa1* [7]. Thus vitamin D serves to limit osteoblast differentiation, in addition to its effects on mineral transport and osteoclast differentiation, and has effects that redirect and sometimes counteract those of other important signals.

Other hormones

Leptin, prostaglandins, glucocorticoids and the sex steroids, especially oestrogen, have major effects on the skeleton. These hormones affect differentiation of bone-forming and bone-degrading cells by complex mechanisms. In general, these hormones amplify or inhibit the fundamental biochemical and cell differentiation processes in the skeleton. Leptin regulates bone mass, apparently by indirect mechanisms through other hormones [58]. Prostaglandins, particularly prostaglandin E₂, have ill-defined roles in modulating bone turnover. Studies *in vitro* indicate that prostaglandin synthase promotes osteoclast formation [59]. However, these powerful regulators of the skeleton affect both osteoclast and osteoblast function, but appear to be dispensable to the essential processes of skeletal formation and turnover. Rather, they have accessory functions that modify the main processes; for example, as sex-specific differences in skeletal shape. Glucocorticoids are important in differentiation and survival of mesenchymal cells, and, in pharmacological concentrations, dramatically increase osteoblast and osteocyte apoptosis [60]. The fundamental processes of bone formation and resorption do not require oestrogen. However, several oestrogen-binding proteins are present in bone, including oestrogen receptors α and β , and many parameters of bone-matrix formation and degradation are altered by oestrogen. Knockout mice deficient in oestrogen receptors α and β have been produced, and both show altered bone mass and turnover (reviewed in [61]). Steroid hormone effects are particularly important when the hormones occur in pharmacological quantities, or where hormonal production declines, such as in post-menopausal women. In these cases, profound and often disastrous effects on skeletal mass occur, by mechanisms that include accelerated apoptosis in regions of the skeleton subject to stress [62].

MECHANISM OF MATRIX DEGRADATION BY OSTEOCLASTS

In contrast with uncertainties regarding bone mineral secretion, the central mechanisms that mediate the removal of bone mineral by the osteoclast are well defined (Figure 3A). Monocytic precursors fuse to form osteoclasts, a process controlled by contact-mediated signalling between osteoblasts and osteoclasts in terminal stages of normal osteoclast differentiation [63]. An essential feature that differentiates the osteoclast from other macrophage polykaryons (giant cells) is massive expression of a cell-surface, vacuolar-like H^+ -ATPase [64], the driving force for acid secretion, which dissolves bone mineral. The protons are derived from CO_2 via carbonic anhydrase II [65]. The H^+ -ATPase and an electrically coupled ClC-7 Cl^- channel [66,67] produce HCl. Cellular acid–base balance is maintained by a $\text{Cl}^-/\text{HCO}_3^-$ exchanger on the basolateral cell membrane [68].

Figure 3 Osteoclast matrix degradation and regulatory mechanisms

(A) The osteoclast acid degradation mechanism. A vacuolar-like H^+ -ATPase, carbonic anhydrase II and a ClC-7 chloride channel electrically coupled to the electrogenic ATPase are, along with a Cl^-/HCO_3^- exchanger that maintains pH balance, mediators of acid secretion. Osteoclasts also secrete acid phosphatase and thiol proteinases. The extracellular compartment is formed by processes including $\alpha_v\beta_3$ integrin binding. Degraded calcium and collagen fragments are removed by vacuolar transcytosis. (B) Selected regulatory mechanisms. In addition to receptors that mediate cell differentiation and apoptosis, signals that regulate osteoclastic activity or survival include Ca^{2+} and nitric oxide. Nitric oxide is produced by osteoblasts; nitric oxide and superoxide are also products of macrophage family cells and are autocrine stimuli under some circumstances. Nitric oxide mediates most of its cellular effects via cGMP, produced in the osteoclasts by a soluble guanylate cyclase. cGMP activates a kinase that down-regulates acid secretion. The osteoclast is exposed to very high calcium at its acid secreting site. Calcium fluxes in the osteoclast include local bursts of uncertain origin and regulated transport by a type-2 ryanodine receptor. Calcium activates calmodulin-dependent processes, including cGMP degradation and phosphatase activity via calcineurin, which has several isoforms. Calcium-dependent processes often act in opposition to nitric-oxide-dependent processes, although at high concentrations either stimulus can mediate apoptosis. Additional regulatory processes interact with calcium and kinase signals.

Osteoclasts secrete a tartrate-resistant acid phosphatase and thiol proteinases, including an acidic collagenase, cathepsin K, that is required for efficient bone degradation [69]. The osteoclast forms a tightly sealed compartment at its bone attachment that allows the extracellular acidification. This sealing process depends on the $\alpha_v\beta_3$ integrin [70]. Degraded calcium and collagen fragments are transported through the osteoclast by vacuolar transcytosis [71,72].

Osteoclast developmental regulation

The response of osteoclasts and their precursors to developmental and regulatory signals is complex. Key developmental and regulatory signals are driven by tyrosine kinase and TNF receptors. Colony-stimulating factor-1 (CSF-1), the ligand for the membrane tyrosine kinase receptor *fms*, is a uniform requirement for monocyte differentiation and is probably required for osteoclast formation and survival in all species. CSF-1 exists in membrane-bound and soluble forms [73]. It is produced by many mesenchymal cells, including osteoblasts. In early differentiation, CSF-1 drives the nuclear transcription factor PU.1, an absence of which prevents osteoclast differentiation [74]. The CSF-1 gene is mutated in an op/op mouse, and osteoclasts fail to differentiate [75]. Src is an intracellular membrane-anchored tyrosine kinase that is a secondary signal for tyrosine kinase receptors and many other signals. Src is one of a family of cytoplasmic tyrosine kinases with overlapping expression and function. However, src knockout mice have dysfunctional osteoclasts [76]; the detailed mechanism of this dysfunction is unknown.

Role of TNF-family proteins in bone turnover

In CSF-1-primed rodent marrow monocytes, the TNF family signal RANKL induces osteoclast differentiation. Mice with their RANKL ablated have osteopetrosis [77]. Thus RANKL is unique in its ability to drive terminal osteoclastic differentiation as a single signal. However, transformation of monocytes to osteoclasts by soluble RANKL *in vitro* is typically inefficient. Whether additional signals are involved *in vivo*, or if this variability is related to competing differentiation stimuli, is unknown. RANKL is produced by T-lymphocytes and by many mesenchymal cells outside of the bone [78], where osteoclasts do not occur, so osteoclast differentiation is not permitted under most circumstances where monocytes are exposed to RANKL; the mechanisms limiting osteoclasts to bone are unknown. Nevertheless, RANKL, or the closely related protein TNF α [79], are implicated as requirements for terminal osteoclast differentiation in nearly all circumstances. Most exceptions are probably related to autocrine TNF α production, which occurs when monocytic cells contact foreign surfaces, such as in tissue culture. In addition to the RANKL receptor, i.e. RANK, osteoclast differentiation is promoted by TNF receptors, including CD95 [80]. TNF α is also an important regulator of osteoblast apoptosis [81], highlighting the connection of osteoblast removal and osteoclast formation.

Inhibitors of late osteoclast differentiation

The TNF receptor family includes soluble receptors that have broad TNF-binding capabilities, which act to limit signalling. These include osteoprotegerin, and the decoy receptors DcR1/TRID, DcR2/TRUNDD and DcR3. All of these bind the TNF-

related apoptosis-inducing ligand, TRAIL [82,83]. Osteoprotegerin also binds RANKL, and blocks osteoclast formation [77]. Numerous studies have indicated that osteoprotegerin is a physiological regulator of osteoclast activity, including the demonstration that absence of osteoprotegerin leads to osteoporosis in rodents [84].

Shared signalling pathways for osteoclast differentiation

RANK activates NF- κ B, interacts with multiple TNF-receptor-associated factors (TRAFs), including TRAFs 2, 5 and 6, activates the Jun kinase, and interacts with Src-related signals and smad3 [85–89]. NF- κ B is present in the cytoplasm as an inactive complex with inhibitor κ B ('I κ B'). Kinase activation by IL-1, TNF α and RANK leads to the cytoplasmic phosphorylation of I κ B and its ubiquitin-related degradation, allowing nuclear localization of NF- κ B [90]. Acting via TRAF-6, IL-1 activates pathways replacing the late stages of RANK signalling [91]. The TRAF pathways are also activated by TNF α [92]. This convergence on NF- κ B emphasizes its role in osteoclast differentiation. There are two forms of NF- κ B that have redundant functions. When both are knocked out, a defect in osteoclast differentiation and osteopetrosis occurs [93].

Regulation of osteoclast formation

Because of the multifunctional role of bone degradation, many additional hormones and cytokines regulate osteoclast formation. These factors, including 1,25-dihydroxyvitamin D, prostaglandin E2 and parathyroid hormone, act primarily on the osteoblast and affect cbfa1-dependent transcription of proteins that also regulate osteoblastic proliferation, matrix synthesis, and survival. These hormones and cytokines modify the expression of CSF-1 or RANKL, the key osteoclast differentiation signals discussed above. Additional cytokines including IL-1, IL-6 and tyrosine kinases related to Fms in some circumstances are expressed highly in bone. IL-1 and IL-6 enhance osteoclastic activity. Their production is co-ordinated with RANKL or TNF α in stromal cells [94], and is stimulated by PTH [95]. IL-1, IL-6 and TNF α are produced by stromal cells, macrophages, osteoclasts and osteoblasts, and thus may be autocrine or paracrine stimuli [96]. IL-6 induces osteoclast precursors to differentiate by acting on gp130 [97]. The IL-1 decoy receptor, IL-1RII, can limit expression of pro-apoptotic TNF family proteins by monocyte family cells that are involved in arthritic destruction of joints [98]. Met and Kit are tyrosine kinase receptors related to Fms, which are also expressed in the osteoclast and its precursors [99,100]. The ligands for these receptors are secreted by osteoblasts, sometimes massively [101], but their physiological functions are uncertain.

Regulation by Ca²⁺ and matrix flexion

Bone is active in rapid calcium and pH regulation where changes occur without increases in the number of cells, by modulating the transport activity or survival of mature cells. For the most part, this involves reduced or increased osteoclastic mineral mobilization, and is reflected in circulating calcium. In addition, there are protective mechanisms, particularly relating to skeletal flexion, that limit bone degradation.

Calcium signalling mechanisms

Osteoclasts, as calcium-mobilizing cells, not surprisingly have an active calcium regulatory system, including a membrane Ca²⁺-

ATPase [102]. They are exposed to high Ca²⁺ (8–40 mM) in the resorptive low pH environment (Figure 3B). Exposure of osteoclasts to similarly high levels of ambient Ca²⁺ *in vitro* results in an immediate rise in cytosolic Ca²⁺. This is associated with cell-matrix detachment, margin retraction, reduced enzyme secretion and reduced bone resorption [103,104]. This is likely to represent a negative feedback mechanism, through which an osteoclast can regulate its own activity using Ca²⁺ as both extracellular and intracellular signals. However, a high extracellular calcium concentration can also elevate the synthesis and secretion of the pro-resorptive cytokine, IL-6 [105]. IL-6, in turn, inhibits Ca²⁺ sensing, thus releasing the osteoclast from Ca²⁺-induced inhibition via an as-yet-uncharacterized mechanism. Ryanodine receptor type 2, expressed uniquely at the osteoclast plasma membrane, appears to function as the Ca²⁺ sensor, as well as a channel for Ca²⁺ influx [106]. The activity of the ryanodine receptor appears to be regulated by Ca²⁺ and the cellular second messenger, cADP-ribose [107]. cADP-ribose production from the intermediary metabolite, NAD, is catalysed by a multifunctional cyclase, CD38, representing a novel mechanism via which the cellular metabolism of a resorbing osteoclast is intricately linked to its Ca²⁺ regulatory mechanism [108].

Additionally, osteoclast and osteoblast endoplasmic reticular membranes and inner nuclear membranes possess type 1 ryanodine receptors, Ins(1,4,5)P₃ receptors and CD38. In this way, it is likely that nucleoplasmic Ca²⁺ levels that effect gene transcription and apoptosis are regulated separately from cytosolic Ca²⁺ [109].

In addition to the nuclear and plasma membrane calcium regulation, osteoclasts have oscillations or spikes in intracellular calcium, which may reflect directly the high-calcium, low-pH resorption compartment that is the main function of the cell [110]. Small calcium fluxes are likely to be important in maintaining bone resorbing activity. In contrast, very high extracellular [Ca²⁺] inhibits osteoclastic bone resorption, and this occurs by alterations in actin organization and podosomal disassembly [111]. Alterations in actin organization probably occur through the suppression of the tyrosine kinases Src and the focal adhesion kinase ('FAK'), since inhibitors of tyrosine kinases repress actin organization and osteoclastic resorption activity [112].

Flexion, cation transport and nitric oxide

Skeletal flexion is a primary stimulus important to the balance of bone formation and degradation, which increases bone synthesis and reduces osteoclastic activity in the region flexed. Mechanical transduction is typically mediated by stretch-responsive cation channels, and there is evidence that this is the mechanism in the osteoblast [113]. The linkage of these signals to changes in bone synthesis and degradation is not known, but probably involves mainly modification of the osteoblast and osteoclast differentiation signals in regions of skeletal flexion. Stretched osteoblasts display enhanced proliferation and bone-matrix synthesis [114], and produce the osteoclast down-regulating signal, nitric oxide [115] (Figure 3B). Other effects of stretching on osteoblasts are reported, but their physiological roles are unclear. The osteoblast is probably the major source of NO in bone under most conditions, and NO synthesis is also stimulated by cytokines, including TNF α [116]. However, under some conditions, NO is produced by osteoclasts [117].

Nitric oxide is probably of particular importance in reducing osteoclastic activity. The effects of NO on osteoclasts are mediated by an NO-dependent guanosyl cyclase, and the principal effects of moderately increased cGMP are mediated by a cGMP-dependent protein kinase [118], although cGMP-inde-

pendent mechanisms are also reported [119]. Furthermore, NO-mediated signals may be bidirectional [120], and, at high concentrations, NO and calcium mediate apoptosis of osteoclasts and precursor cells [121,122]. NO and calcium can also regulate osteoclastic activity without causing apoptosis, depending on the size of the stimulus and other factors [121,123]. These pathways often act in opposing directions.

APOPTOSIS IN BONE DEVELOPMENT AND MAINTENANCE

Programmed cell death is evident in all stages of skeletogenesis and maintenance. During development, segmentation (Figure 1) and cartilage replacement (Figure 2) are instances of massive overt cell death. Even though apoptosis in the skeleton is a complex subject that is incompletely understood, general patterns have emerged that provide a useful conceptual framework. Regional apoptosis in skeletal development and in turnover follows the pattern of development, and in these instances apoptosis is triggered by withdrawal of stimuli such as BMPs, although hormones and cytokines modify the rate and extent of this process. Degradative cells are recruited by cytokines in the tumour necrosis family, including RANKL and TNF α , that drive apoptotic and inflammatory pathways, with suppression of apoptosis generally depending on secondary stimuli. This general scheme is illustrated in Figure 4(A).

Skeletal apoptosis recapitulates morphogenic mechanisms

Apoptosis is the central mechanism for segmentation of primordial skeleton to produce individual bones. This site-directed apoptosis is driven by cytokine withdrawal that includes the

TGF- β -BMP and FGF families, with the former typically exerting primary control [17]. The molecular mechanism of withdrawal can involve decoy receptors (inhibitors), as well as regulated expression [15,19,20]. FGF family cytokines are prominent where interactions with epithelial cells occurs [21]. The mineralization of cartilage and formation of cellular bone requires orchestrated removal of chondrocytes resulting as the cytokine support is reduced and other cells differentiate [25]. In mature bone, under most circumstances BMP2 is present as an autocrine factor [124] and the apoptotic decision remains critically dependent on FGF signalling [22]. The initiation of cell death results from complex interactions. For example, there is evidence that, following terminal differentiation, BMP2 becomes pro-apoptotic unless suppressing signals including TGF- β are also present [125]. Additionally, signals such as the TNF- α and inflammatory cytokines can modify, in most cases promoting, osteoblast apoptosis [126].

Subsequently, the mineralization of the cellular bone requires the removal of effete chondrocytes and mineralized cartilage, and (later) of bone matrix during turnover. Signals involved in this process include TNF- α , as well as IL-1 and other 'inflammatory' cytokines. These, together with RANKL, also mediate osteoclast formation, and osteoclast formation is enhanced by inflammatory cytokines, including IL-1. Typically, these signals, in contrast, promote osteoblast apoptosis [92]. In this way, opposing regulatory signals can determine a switch from synthesis to degradation.

Other counter-regulatory systems include secondary suppression of TNF-type apoptosis by CSF-1 in the osteoclast [127], which appears to be a requirement for growth factors to delay the TNF-driven death decision in osteoclasts. Inside the os-

Figure 4 Apoptosis in osteoclasts and osteoblasts

The diagrams shown include intermediate steps generalized from other cells, as specified in the text. (A) Caspase and mitochondrial pathways for apoptosis. Stimuli for osteoclast apoptosis include a balance of death receptor and suppressing cytokines. Tandem activation of caspase and BCL-2 proteins in osteoclasts is demonstrated, which may act in both bone-forming and -degrading cells (with different stimuli). Proximate stimuli include the indicated receptors, as well as calcium, steroids and NO receptors. The intermediate macromolecular complexes in these cells are not well studied. Proceeding downstream from an initiating signal, here illustrated as originating from a TNF receptor, apoptosis manifests tandem execution phases, effector caspase activation and organelle dysfunction. The caspases are proteinases that take the committed cell to an organized demise by cleaving other caspases and by activating proteins that cause organelle dysfunction. The BCL-2 family. Mitochondrial dysfunction, defined by the action of pro-apoptotic BCL-2 family proteins, is an important point in the process; if this decision is passed, it is not possible to rescue the cell. The pathways illustrated include elements detected during apoptosis in bone, but physiological mechanisms will be more complex. Particularly, inhibiting proteins and alternative activators may be present and are not illustrated. (B) Apoptosis and bone-forming units. In the osteoblast, apoptosis may be driven by withdrawal of survival factors of the TGF- β family or by stimuli including TNF. Osteoblasts are connected by gap junctions and respond to cytokines and death signals, in groups; such an event triggered by a hypothetical massive calcium release related to organelle dysfunction is illustrated schematically. Although this pathway is hypothetical, gap junctions transmit calcium, and calcium triggers apoptosis. A TUNEL (terminal transferase deoxynucleotide end labelling) assay showing apoptosis of bone cells in groups within the trabecular bone of rabbit femora of a glucocorticoid treated rabbit is shown at the right of the diagram. The apoptotic bone cells occur in clusters. At this stage, the surface osteoblasts have been removed and the surface is irregular due to osteoclastic activity.

teoclast, the mechanisms involved in suppression of apoptosis include stimulating the relative activity of extracellular-signal-regulated kinase ('ERK') relative to NF- κ B transcription factors, which antagonize each other, the latter being pro-apoptotic [128]. More general enhancing factors for osteoclast apoptosis include glucocorticoids [129] and oestrogens [130], although the mechanisms of these effects are not established. Additional mechanisms that modulate apoptosis in osteoclasts certainly exist, although the importance of many factors studied *in vitro*, and how they interact, is unclear. Calcium and nitric oxide (discussed above) are clearly physiologically important, however.

Caspase and mitochondrial death pathways

Biochemical and biophysical processes culminating in a death 'decision' are similar for all metazoans. It is clear that commitment to cell death is dependent upon organelle dysfunction, which frequently is manifested as mitochondrial membrane disruption [131]. Proximate activation of apoptosis can be a positive stimulus or result from withdrawal of cytokine, indicating a default death decision.

Caspase activation in bone

The caspases are pro-enzymes that, when activated, cause processing of cellular components to a non-necrotic and organized demise. In osteoblasts, as in many other cells, a decision to initiate apoptosis is induced by a variety of stimuli, including withdrawal of signals upon which the cell is dependent, as well as hormones, calcium and TNF α , which activates caspase-3 [132–134]. It is not clear where BMP- or FGF-related proteins influence this common pathway, but at high concentrations BMP2 becomes pro-apoptotic [125]. In osteoblasts, calcium-mediated co-ordination of apoptosis is probably important because of the gap junctions that connect functional osteoblastic units (the osteons), although this mechanism is not confirmed experimentally (Figure 4B).

Organelle dysfunction and the Bcl-2 protein family

The important proximal decision to proceed to cell death is defined by the action of the Bcl-2 family of proteins [135]. Once this decision is transmitted it is no longer possible to rescue the cell by inhibition of the caspases. Mitochondrial dysfunction has been extensively studied, since it represents a paradigm for organelle dysfunction in the control of programmed cell death. Bcl-2 family proteins are associated with intracellular membranes [136], including mitochondria, endoplasmic reticulum and nuclear membranes. The three-dimensional structure of Bcl-X_L showed conformational similarity to the membrane insertion domains of bacterial toxins, colicins and diphtheria [137]. Subsequently, a number of laboratories have demonstrated that Bcl-2 family proteins are incorporated into bilayer membranes and generate water-filled diffusion pathways across membranes [138–141]. Further attention was focused on membrane pores with the report that cytochrome *c* is released and is sufficient to mediate caspase-9 activation and late stages of apoptosis [142]. The activity for cytochrome *c* transport resides in pro-apoptotic Bcl-2 family proteins [143], but the regulation and activation of these molecules remains to be clarified.

Bcl-2 family proteins and apoptosis in bone

Pro- and anti-apoptotic Bcl-2 family proteins are expressed in apoptotic cartilage and bone [34,144], and subpopulations of osteoblasts with different Bcl-2 protein expression can be discrim-

inated [145]. The functional consequences of these differences remain to be determined. With respect to osteoclasts, continued signalling by both CSF-1 and RANKL prevent apoptosis [146]. Transgenic animals with enhanced Bcl-X_L function have greatly increased osteoclastic function [147]. Serum withdrawal causes apoptosis of osteoclasts, which is opposed by IL-1 or CSF-1 [148].

THE GENERAL MECHANISM OF SKELETAL TURNOVER

In mature animals, skeletal repair and turnover recapitulate processes that appear in development and morphogenesis, where apoptosis and macrophage-derived degradative cells separate skeletal elements that are produced by bone- or cartilage-synthesizing cells. There are several gaps in understanding, including the molecular mechanism of cytokine control of apoptosis involving both activation and inhibition of cell death. Furthermore, the skeleton interacts with other organs, requiring co-ordination with muscular, circulatory and neural development and metabolism. Additionally, bone is the major reservoir of calcium and pH equivalents. For all these reasons, the control of skeletal biochemistry is complex. However, it is clear that differentiation and apoptosis of mesenchymal cells in a dynamic balance with bone-degrading cells is a valid central principle. This balance, in turn, regulates synthesis and degradation of the skeletal matrix.

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