Apoptosis in bone physiology and disease

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Bone is typically thought to provide support for muscles and protection for vital organs but to be rather inactive at the cellular level. Thus, the concept of cell death in bone being of any importance to the physiology or pathology of this tissue, at first glance, may seem strange. However, the reality is that bone is a dynamic tissue the cells of which are involved in complex interactions with one another and with haemopoietic and stromal cells in bone marrow in a process that continually remodels the skeleton. Our current understanding is that two classes of cells are involved in this process, osteoclasts, which resorb bone matrix, and osteoblasts, which synthesise new bone matrix. These two families of cells interact at sites in bone where turnover is actively occurring at locations in periosteal, endosteal trabecular, and cortical bone. These sites are referred to as basic multicellular units (BMU). Within each BMU bone is resorbed by osteoclasts that then disappear and are replaced by osteoblasts which resynthesise bone, some becoming osteocytes. This sequence of cellular events is described extensively elsewhere.^{1,2}

There are several situations under which cell death may occur in bone as a physiological event (table 1). Probably the best recognised of these is during ossification of the growth plate. Hypertrophic chondrocytes disappear as growth plate cartilage calcifies and is resorbed from the primary spongiosa. Recent evidence has suggested that hypertrophic chondrocytes undergo apoptosis at this site.³ As the balance of evidence suggests that osteoclasts usually disappear at the cessation of their resorptive activity, it would seem likely that they undergo some form of cell death. In addition, it is conceivable that regulation of numbers of osteoclast precursors through induction of cell death may represent a mechanism by which the number of mature osteoclasts at any site is controlled. Whether cell death is an important event in the control of the activity of the osteoblast lineage is more difficult to gauge. From a theoretical perspective, it is possible that the number of mature osteoblasts that form in any BMU depends upon the balance of mitosis and apoptosis of their marrow stroma derived precursors. Also, although there are two well defined fates for osteoblasts (differentiation into osteocytes or lining cells), it is also possible that some osteoblasts undergo cell death. The remainder of this article provides an overview of what is currently known about cell death in the osteoclast and osteoblast lineages, and how the death of these cells may be of relevance to clinically important bone diseases.

Cell death: general considerations

Cell death is a surprisingly complex process that has received much study during the 1990s, having been all but ignored during the previous century. In simple terms, cells can be murdered (necrotic cell death) or commit suicide (apoptotic cell death). The complexities of cell death have been eloquently reviewed elsewhere,^{4,5} and the distinction between necrotic and apoptotic cell death is becoming increasingly familiar to the general scientific audience. Apoptosis represents the "physiological" mode of cell death and is therefore of relevance to the normal cellular events of bone. The important characteristics of apoptosis are: (1) apoptosis consists of a fairly stereotyped sequence of morphologically recognisable events; (2) apoptosis is controlled from within the cell by a large number of regulatory factors, but can be induced or inhibited by external factors through receptor mediated mechanisms.

Osteoclast apoptosis

REGULATION OF OSTEOCLAST ACTIVITY

The osteoclast is a rare and elusive cell. Osteoclasts can be found at any of the million or so active remodelling sites that exist at any one time in the adult human skeleton, but occupy a very small proportion of the total bone surface. The osteoclast is extremely rapid and effective in destroying bone matrix, therefore its activity has to be tightly regulated to maintain normal skeletal homeostasis. The potentially disastrous consequences of loss of regulation of osteoclast activity are all too apparent in lytic bone diseases such as myeloma. The regulation of osteoclast formation and bone resorption has received considerable study (reviewed by Mundy and Roodman⁶). It is now well known that osteoclasts are derived from haematopoietic precursors that appear to be phenotypically indistinguishable from the precursors of the monocyte/macrophage lineage until a very late stage in their differentiation, at which time they begin to acquire characteristics of adult (multinucleated, bone resorbing) osteoclasts such as expression of tartrate resistant acid phosphatase (TRAP) and calcitonin receptors. The proliferation and differentiation of the precursors is controllable by a wide range of factors including systemic hormones such as parathyroid hormone (PTH), 1,25-dihydroxyvitamin D₃ (1,25D) and sex steroids, and cytokines/growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), and interleukin (IL)-6. Mature osteoclasts are thought to lack receptors for most of the factors capable of stimulating or inhibiting

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Table 1 Cell death in bone physiology

Cell type	Cell death has been demonstrated*	Cell death may occur†
Growth plate chondrocytes	During ossification of hypertrophic zone	
Osteoblast precursors		As a mechanism for limiting osteoblast recruitment
Osteoblasts		At the cessation of bone formation
Osteocytes		Before bone resorption
Lining cells	During the reversal phase of bone remodelling	At the initiation of resorption
Osteoclast precursors		As a mechanism for limiting osteoclast recruitment
Osteoclasts		

*Demonstrated in vivo in genetically normal animals; †speculative possibilities, some of which have been suggested as interpretations or justifications for studies cited in this review.

their resorptive activity,^{7, 8} but this is controversial, in part because of the lack of pure osteoclast cell preparations or cell lines. Experimental evidence suggests that once osteoclasts have formed and are resorbing bone, the resorptive activity of individual osteoclasts is controlled by soluble mediators produced by other cells, probably cells of the osteoblast lineage.^{7, 8} However, another possible mechanism by which osteoclast activity could be controlled is by lengthening or shortening the lifespan of individual osteoclasts. As the majority of nucleated cells appears to possess the genetic programming to undergo programmed cell death (apoptosis), and that the apoptotic programme can be turned on or off by physiological stimuli, it is theoretically possible that osteoclast activity could be controlled through regulated apoptosis. For this to be the case, apoptosis would have to be the normal fate of the osteoclast.

FATE OF THE OSTEOCLAST

The fate of the osteoclast has been speculated upon intermittently for 100 years. The subject was reviewed in 1920 by Arey⁹ who proposed that osteoclasts disappear from the bone surface after undergoing degenerative anatomical changes. More recently, the possible fates of osteoclasts during the reversal phase of bone remodelling were reviewed by Baron and co-workers² who suggested that osteoclasts might undergo fission to form the mysterious macrophage-like cells that have been described at bone surfaces during reversal. Subsequently, Liu and co-workers¹⁰ studied the rapid loss of osteoclasts from the bone surfaces of calcium deficient rats following calcium repletion, and speculated that osteoclasts were undergoing cell death in this particular situation. Osteoclast apoptosis was first identified in vitro by Fuller and co-workers, who found that isolated rat osteoclasts underwent apoptosis if not treated with M-CSF,¹¹ and in vivo by Boyce and co-workers in a strain of transgenic mice.¹² In these mice, SV40 T antigen was under the control of the TRAP promoter, resulting in abnormal proliferation of osteoclasts and their precursors. Rather than developing osteoporosis, as might be expected because of the increased numbers of osteoclasts, these animals developed osteopetrosis (increased bone density). Unexpectedly, many osteoclasts showed the morphological features of apopto-

sis, an observation that was supported by a DNA strand end-labelling technique designed to detect the ends of DNA fragments resulting from the endonuclease activity characteristic of apoptosis.^{13, 14} These observations indicated that increases in osteoclast apoptosis may reduce bone resorption (hence the development of osteopetrosis in these mice) and raised the question of whether apoptosis might be a physiological event in the life cycle of the osteoclast. The question of the fate of normal osteoclasts was addressed by histological examination of calvarial bones from mice treated with local injections of recombinant IL-1. In this model, IL-1 causes intense, localised bone resorption that is followed by abrupt cessation of resorption and new bone formation several days after the cessation of IL-1 injections.¹⁵ Histological examination of bones taken on the fourth day after the last IL-1 injection revealed a very high proportion (up to 13%) of osteoclasts showing the morphological features of apoptosis, namely loss of adhesion to the bone surface, cytoplasmic contraction and blebbing, nuclear chromatin condensation, and nuclear fragmentation.¹⁶ The osteoclasts showing these characteristics were very rarely situated at active resorption fronts, usually being at sites of reversal between areas of active resorption and active bone formation.

These observations strongly suggest that apoptosis is the normal fate of osteoclasts at reversal, and have subsequently been supported by a study in which the calcium repletion model of Liu and co-workers¹⁰ was revisited to show that the rapid loss of osteoclasts from the bone surface in these animals was associated with apoptosis.¹⁷

REGULATION OF OSTEOCLAST APOPTOSIS

Methods used to study osteoclast apoptosis

The study of osteoclast apoptosis has been complicated by the comparative rarity of this cell type and the short duration of morphologically recognisable apoptosis. It is also difficult to isolate large numbers of osteoclasts in vitro and it is impossible to achieve a 100% pure population of these cells. However, a characteristic feature of the osteoclast is that it continues to express TRAP strongly during apoptosis (fig 1b and c). Of the cells normally present in bone marrow, only the osteoclast strongly expresses TRAP.¹⁸ This ensures that apoptotic osteoclasts can be identified readily in tissue sections and in vitro, thus facilitating the development of techniques for studying osteoclast apoptosis quantitatively both in vivo and in vitro.¹⁹ This morphological approach was validated by using other techniques for detecting apoptosis, namely DNA strand end-labelling and acridine orange staining.^{13, 19} Furthermore, some established techniques used to study bone resorption and osteoclast formation in vitro may indirectly measure osteoclast survival. For example, bone resorption occurring in an ex vivo bone resorption assay using neonatal mouse calvariae reflects osteoclast survival, rather than osteoclast recruitment or activation, after the first 24 hours of culture.²⁰ In addition, IL-1 and M-CSF have been shown to prolong the survival of cultured

Figure 1 Examples of cell death occurring in bone. (a–c) Osteoclast apoptosis. (a) Histology from a giant cell tumour of bone demonstrating an apoptotic osteoclast (solid arrow) with a viable osteoclast (open arrow) for comparison. The apoptotic cell shows the chromatin condensation, nuclear fragmentation, and cytoplasmic contraction typical of apoptosis. (b) Apoptotic osteoclast stained for tartrate resistant acid phosphatase (TRAP; red cytoplasmic staining), with a similarly stained viable osteoclast in (c) for comparison. In addition to the features of apoptosis demonstrated in (a), the apoptotic osteoclast shows detachment from the bone surface and cytoplasmic fragmentation. (d,e) Osteocyte death. (d) Subchondral bone from a hip joint affected by osteoarthritis. The majority of osteocyte lacunae (arrows) are empty. In contrast, osteocytes can be seen in the majority of lacunae in subchondral bone from a normal hip (e). The mechanism leading to the apparent loss of osteocytes in osteoarthritis is unknown. (a,d,e) Haematoxylin and eosin; (b,c) TRAP/haematoxylin; all original magnification $\times 400$.

osteoclasts, possibly through the suppression of apoptosis,²¹ as osteoclasts appear to be prone to die by apoptosis *in vitro*.^{11 22–25}

Effects of calcitropic hormones and cytokines on osteoclast apoptosis

The techniques referred to have been used to study the regulation of osteoclast apoptosis by a variety of calcitropic hormones and cytokines. In general, factors that stimulate bone resorption inhibit osteoclast apoptosis and factors that inhibit bone resorption promote osteoclast apoptosis. There are exceptions—calcitonin, which inhibits bone resorption, does not affect osteoclast apoptosis,²² an observation that is in keeping with the reversibility of this hormone's action.²⁶ Current knowledge of the regulation of osteoclast apoptosis by hormones and cytokines is detailed in table 2. These data suggest that regulation of osteoclast apoptosis may account, at least in part, for the mechanism by which these factors regulate bone resorption, particularly in the case of cytokines such as IL-1 and TNF α .

Effects of oestrogen on osteoclast apoptosis

Perhaps the most interesting of the observations listed in table 2 is that the oestrogen, 17 β -oestradiol (E_2), promotes osteoclast apoptosis both *in vitro* and *in vivo* in ovariectomised mice.²⁷ Similarly, the oestrogen agonist/antagonist tamoxifen, which has oestrogenic effects in bone, promotes osteoclast apoptosis *in vitro*.^{25 27 28} The implication of this observation is that promotion of osteoclast apoptosis may be the mechanism by which E_2 inhibits bone resorption in post-menopausal osteoporosis, and conversely that a failure of apoptosis may, at least in part, account for the increased osteoclast activity seen in post-menopausal osteoporosis. Although there is at present no direct evidence to support the latter hypothesis, there is growing evidence that cytokines such as TNF α , IL-1, IL-6, and M-CSF mediate the increased osteoclast number and bone resorption that follow oestrogen withdrawal.^{29–31} All of these factors inhibit osteoclast apoptosis *in vitro*.^{11 32} (Hughes and Boyce, 1995 unpublished observations). There is, however, persuasive evidence that oestrogens can influence osteoclast formation. Bone marrow from ovariectomised mice gave rise to greater numbers of osteoclasts in *ex vivo* cultures than did marrow from sham operated animals, and this effect was reversed by oestrogen treatment.³¹ Study of oestrogen treated murine marrow cultures revealed that, in addition to an increase in osteoclast apoptosis, there was an increase in apoptosis of TRAP positive mononuclear cells (Hughes and Boyce, 1995 unpublished observations). The precise identity of these cells is not certain but it is possible that they represent committed osteoclast precursors. This suggests that oestrogens may also reduce osteoclast formation by depletion of their precursors; this is supported by studies showing that oestrogens can increase apoptosis in mixed bone marrow preparations³³ and that E_2 and an oestrogen agonist (CP-336,156) inhibited osteoclast formation by an apoptotic mechanism.³⁴

Table 2 Regulation of osteoclast apoptosis

Factor	Effect on osteoclast apoptosis	
	In vitro	In vivo
Hormones		
Parathyroid hormone	Inhibits	Unknown
1,25D	Inhibits	Unknown
Calcitonin	No effect	Unknown
Corticosteroids	Stimulates	Unknown
17β-oestradiol	Stimulates	Stimulates
Testosterone	Stimulates	Stimulates
Cytokines		
M-CSF	Inhibits	Unknown
IL-1	Strongly inhibits	Unknown
TNFα	Strongly inhibits	Unknown
IL-6	Inhibits (when combined with its soluble receptor)	Unknown
TGFβ	Stimulates	Stimulates
Drugs		
Bisphosphonates	Strongly stimulates	Strongly stimulates
Tamoxifen	Stimulates	Unknown
RGD peptides (antagonistic to α,β, integrin)	Stimulates	Unknown

1,25D, 1,25-dihydroxyvitamin D₃; M-CSF, macrophage-colony stimulating factor; IL, interleukin; TNF, tumour necrosis factor; TGF, transforming growth factor.

Osteoclast–matrix interactions and osteoclast survival

When deprived of attachment to their normal substrate, many types of cell die by apoptosis, a process that has been termed anoikis.³⁵ The osteoclast may be no exception to this. Before undergoing apoptosis at sites of reversal within BMU, osteoclasts lose attachment to the bone surface.¹⁶ This may not be coincidental, as osteoclasts cultured on dentine slices have a lower basal frequency of apoptosis than controls cultured on plastic, and are more resistant to induction of apoptosis by bisphosphonates.¹⁹ In addition, integrin specific inhibitory peptides can induce osteoclast apoptosis, particularly those that are specific to the major osteoclast integrin, α_vβ₃ integrin (the classical vitronectin receptor).³⁶ Whether this results from inhibition of matrix attachment or a more direct receptor–ligand interaction remains to be determined.

Pharmacological stimulation of osteoclast apoptosis: effects of bisphosphonate drugs

Bisphosphonates are well known inhibitors of bone resorption. They are used in the treatment of Paget’s disease of bone, hypercalcaemia of malignancy, and osteoporosis.³⁷ The mechanism of action of these drugs has not been fully clarified. Both in vitro and in vivo studies have revealed that bisphosphonates promote osteoclast apoptosis.^{19–22} This supports the concept that promotion of osteoclast apoptosis may be an effective strategy in treating diseases of increased bone resorption such as post-menopausal osteoporosis. The underlying mechanism of this effect is not known.

Biochemical mechanisms of osteoclast apoptosis

The underlying mechanisms controlling osteoclast apoptosis are currently unknown and will doubtless be the subject of future study. Preliminary studies suggest that transforming growth factor (TGF)β may be important in the action of oestrogen, as the effect of E₂ on osteoclast apoptosis could be reversed by a pan-specific anti-TGFβ antibody.²⁷ There is also evidence that osteoclast–matrix interac-

tions could be important in mediation of osteoclast apoptosis. Loss of adhesion of osteoclasts to the bone surface is also a feature that has been described in oestrogen treated animals.³⁸ Study of the subcellular mechanisms controlling osteoclast adhesion may therefore provide clues to the pathways controlling apoptosis.

Cell death in the osteoblast lineage

There is now growing research interest into apoptosis in cells of the osteoblast lineage (bone marrow stroma derived osteoblast precursors, lining cells, and osteocytes). The osteocyte is a very stable and long lived cell and under normal circumstances the fate of osteocytes in bone that is being resorbed is to be phagocytosed by osteoclasts.³⁹ Phagocytosed osteocytes typically show the morphological features of apoptosis and thus this is likely to be their fate before or during phagocytosis.²⁷ However, recent evidence has been presented that apoptosis can be demonstrated in osteocytes in human bone undergoing high rates of turnover, and that this may signal the onset of resorption.⁴⁰ Furthermore, there is evidence that reduction in oestrogen concentrations in premenopausal women can lead to loss of osteocytes.⁴¹ Osteocytes are certainly known to disappear from ischaemic bone, as seen in avascular necrosis, but presumably this is by necrotic cell death. Loss of osteocytes has also been described in associated with osteoarthritis (fig 1d and e), although the mode of cell death in this situation has not been established. Whether the loss of osteocytes contributes significantly to changes in bone remodelling following oestrogen withdrawal awaits further study. The presence of morphologically apoptotic osteoblasts at active bone forming seams in normal human bone or bone from genetically unmanipulated mice appears to be an extremely uncommon occurrence in the experience of the authors. However, a recent study⁴³ showed that it was possible to demonstrate terminal deoxynucleotidyl transferase dependent nucleotide incorporation into active osteoblasts. This is a phenomenon that can be demonstrated in apoptotic cells, although this technique correlates variably with apoptotic morphology in some cell types,⁴⁴ and on its own cannot be regarded as definitive evidence of apoptosis. Increasingly, the regulation of apoptosis in osteoblast-like cells is being studied in vitro, with evidence of regulation by growth factors and cytokines.^{43–46} However, because of the lack of certainty of which stage of the osteoblast lineage each particular in vitro model corresponds to, the significance of these observations to osteoblast function and the pathogenesis of bone disease is uncertain. Osteoblast apoptosis has been unequivocally demonstrated in vivo in one setting. Rats of the *tl* strain are known to have defective osteoblast function and lose osteoblasts from their bone surfaces, which has recently been demonstrated to be due to apoptosis. This was correlated to a defect in the ability of their osteoblasts to assemble actin stress fibres when in contact with the bone surface.⁴⁷

Relevance of cell death to bone disease

GROWTH PLATE CHONDROCYTE APOPTOSIS

Whether abnormalities of chondrocyte death contribute to the pathogenesis of any of the forms of epiphyseal dysplasia is not certain. One recent piece of evidence suggests hypertrophic chondrocyte apoptosis may be important in growth plate ossification.⁴⁸ Hypertrophic chondrocytes overexpressing a mutated form of the fibroblast growth factor receptor FGFR3, which has ligand independent tyrosine kinase activity, as happens in achondroplastic dwarfism, were more resistant to apoptosis. This suggests that apoptosis of hypertrophic chondrocytes has a permissive role in growth plate ossification.

OSTEOCLAST APOPTOSIS

The aforementioned studies of osteoclast apoptosis have been almost exclusively in animal models, therefore, the contribution of changes in the regulation of osteoclast apoptosis to the pathogenesis of bone disease can only be speculated. Human osteoclasts certainly can undergo apoptosis, having been observed in bone from patients with osteoarthritis, hyperparathyroidism, and Paget's disease (Hughes and Boyce, 1995 unpublished observations). It can also be seen readily in osteoclast rich lesions such as giant cell tumours of bone and tendon sheath and aneurysmal bone cysts (Hughes and Boyce, 1995 unpublished observations; Salter and Reid, 1995 personal communications) (fig 1a). The observation of apoptotic osteoclasts in these situations is possible because of the abnormally large numbers of osteoclasts present. Because of the short duration of apoptosis (as little as one hour in some cell types) and the relative rarity of osteoclasts in normal bone, the probability of observing an osteoclast in apoptosis by chance in normal human bone is extremely small. While it is theoretically possible that in some diseases characterised by increased bone resorption (Paget's disease of bone, hyperparathyroidism, and post-menopausal osteoporosis) there is relative inhibition of osteoclast apoptosis, this hypothesis is impossible to test using currently available techniques. However, it is noteworthy that bisphosphonates, which are effective therapeutic agents in Paget's disease of bone and post-menopausal osteoporosis, promote osteoclast apoptosis not only in rodents¹⁹ but also in humans (Boyce and Hughes, 1996 unpublished observations).

APOPTOSIS IN THE OSTEOBLAST LINEAGE

Currently, the only evidence to suggest that apoptosis of any cells of the osteoblast lineage is an aetiological factor in any form of bone disease is a study by Dunstan and co-workers who found empty osteocyte lacunae more frequently in femoral heads from elderly subjects than from young subjects.⁴⁹ They did not comment on osteocyte apoptosis as the possible cause of the disappearance of osteocytes from these bones, but osteocyte apoptosis in the proximal femur could perhaps be linked in some way to the increase in fragility of bone at this site in the elderly. Osteocyte death has

also been reported in osteoarthritis, but this is perhaps more likely to represent a consequence rather than a cause of the disease. Loss of normal function of p53 and the retinoblastoma gene are strongly associated with the pathogenesis of osteosarcoma through a variety of lines of evidence.⁵⁰⁻⁵² When the function of these two genes is lost simultaneously there is evidence that cell proliferation outweighs apoptosis.⁵³ However, the significance of this to the development of osteosarcomas is unknown.

Conclusions

Although cell death in bone may have been described almost 100 years ago, its possible role in bone physiology and pathology has been largely ignored until recently. Even now, the level of knowledge and understanding of cell death in the osteoclast and osteoblast lineages is rather limited because of the difficulty of studying this process, particularly in vivo. However, there is now increasing evidence to suggest that, at least in the osteoclast lineage, changes in the regulation of cell death may contribute to clinically important diseases of bone such as post-menopausal osteoporosis, and that the induction of osteoclast apoptosis is a potential therapeutic tool for treating these diseases.

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