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Antiresorptive Agents and Osteoclast Apoptosis

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Abstract Antiresorptive agents have proven to be effective therapies for the treatment of bone diseases associated with excessive osteoclast activity. Decreased osteoclast formation, inhibition of osteoclast actions, and reduced osteoclast survival represent mechanisms by which antiresorptive agents could act. The goals of this article are to present the evidence that antiresorptive agents can decrease osteoclast survival through apoptosis, to review the mechanisms by which they are thought to activate the apoptotic process, and to consider whether the actions on apoptosis fully account for the antiresorptive effects. As background, the apoptotic process will be briefly summarized together with the evidence that factors that promote osteoclast survival affect steps in the process. Following this, therapeutic agents that are both antiresorptive and can stimulate osteoclast apoptosis will be discussed. Other bone therapeutic agents that are either antiresorptive or apoptotic, but not both, will be described. Finally, newer antiresorptive compounds that elicit apoptosis and could represent potential therapeutic agents will be noted. J. Cell. Biochem. 101: 1087–1096, 2007.
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Key words: osteoclast; apoptosis; bone resorption

Apoptosis is a physiological process that leads to elimination of cells that are extraneous to the developmental process or have undergone damage that triggers their removal. Apoptosis is contrasted with necrosis based on the characteristics that necrosis is initiated by external damage and results in rapid cell swelling and lysis with a consequent inflammatory reaction and tissue destruction. Apoptosis, in contrast, is a process occurring within the cell, that does not elicit an inflammatory reaction and that results in the fragmentation and removal of the cell by a ordered sequence of events [Kerr et al., 1972]. This has resulted in apoptosis being considered a form of "programmed cell death." The distinction between apoptosis and necrosis was made more than 30 years ago, but it is largely in the past 10–15 years that apoptosis has been extensively studied in relation to developmental and pathophysiological processes and drug actions.

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BIOCHEMICAL AND CELLULAR EVENTS IN APOPTOSIS

Apoptosis is characterized by activation of a cascade of caspases, cysteine proteases requiring aspartate as an amino acid at the cleavage position, which results in fragmentation of chromatin and destruction of cell membranes [Hengartner, 2000]. The process is initiated through two independent pathways. A membrane death receptor pathway can be activated through Fas ligand or TNF, and results in the activation of caspases 8 or 10 which then activate caspase 3. A mitochondrial pathway can be activated through cytokines or other stresses and results in the release of cytochrome C, which activates caspase 9 and caspase 3. Adenine nucleotides are crucial for the maintenance of the integrity of the mitochondrial membrane, and interference with the mitochondrial ATP-ADP translocase results in release of cytochrome C. A family of proteins, the bcl-2 family, which contains both proapoptotic (e.g. Bad, Bax, Bim) and antiapoptotic members (Bcl-2, Bcl-xl), facilitates or inhibits the process. Cytochrome C and proapoptotic bcl family members interact with the cytosolic

protein Apaf-1 to form a molecular complex, the apoptosome, which results in the release of caspase 9 [Schafer and Kornbluth, 2006]. There is considerable regulation within the pro- and antiapoptotic members of the bcl-2 family, evidence that apoptosis is a tightly controlled process. Downstream events that are important for cell survival, including the PI3 kinase/Akt pathway and the s6 kinase pathway impact apoptosis at the level of the bcl family. Recent work has demonstrated that caspases are involved in processes in addition to apoptosis [Nhan et al., 2006], and are likely to be critical for osteoclast differentiation [Szymczyk et al., 2006], suggesting a possible link between differentiation and survival events. Morphological features characteristic of apoptosis are widely used to establish the involvement of the process. These include membrane blebbing, cell shrinkage, and DNA fragmentation [Willingham, 1999]. A highly simplified version showing only the basic outline and steps that have been specifically identified as critical for osteoclasts is shown in Figure 1. Detailed models incorporating the multiple steps that have been characterized are available in reviews [Hengartner, 2000; Bredesen et al., 2006; Schafer and Kornbluth, 2006] and on web sites (e.g. http://www.upstate.com/img/pathways/apoptosis; http://www.calbiochem/apoptosis).

APOPTOSIS IN OSTEOCLASTS; EFFECTS OF PRO-SURVIVAL FACTORS ON THE APOPTOTIC PATHWAY

The osteoblast survival factors receptor activator of NFkB ligand (RANKL) and m-CSF impact the apoptosis pathway through the phosphorylation and inactivation of caspase-9

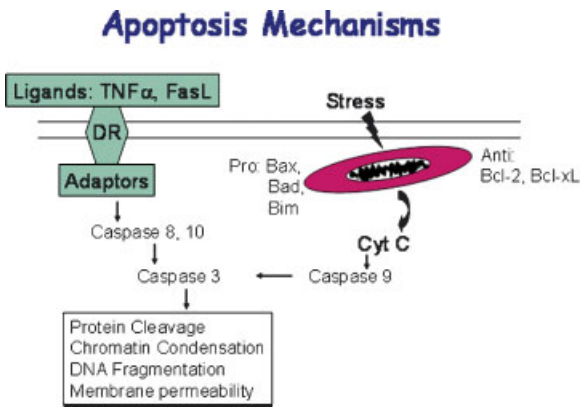

Fig. 1. Basic mechanisms of apoptosis referred to in text. DR = death receptor, cyt C = cytochrome C.

by the kinase Akt [Cardone et al., 1998]. Akt is activated by PI3kinase, which becomes activated after the binding of RANKL to its receptor RANK on osteoclasts [Lacey et al., 2000]. This is followed by recruitment of TNF receptor activator factor 6 (TRAF 6) and the kinase Src. Src signaling also activates nuclear factor κB (NFκB) and Erk pathways, leading to additional downstream survival signaling [Gardner et al., 1993; Imbert et al., 1996]. In cultures of mouse osteoblasts with mouse marrow cells, the Erk pathway was found to be more critical for survival, whereas NFκB was a more important factor for resorption [Miyazaki et al., 2000]. The involvement of Erk in promoting osteoclast survival was demonstrated by the finding that a constitutively active mutant of the upstream kinase Mek 1 promoted survival of osteoclasts [Miyazaki et al., 2000]. PI3K kinase inhibitors and Erk inhibitors prevented the increase in osteoclast survival elicited by TNFα in mature osteoclasts [Lee et al., 2001]. TNFα has mixed actions on the apoptotic pathway in many tissues, being either proapoptotic as shown in the figure, or antiapoptotic. Mammalian target of rapamycin (m-tor) and its downstream kinase S6 kinase are a point at which Akt, Erk, and NF-κB signaling converge in osteoclasts. Inhibition of m-tor/ S6k signaling with rapamycin induced osteoclast apoptosis [Glantschnig et al., 2003].

The proapoptotic family member Bim was shown to be involved in the induction of apoptosis associated with removal of m-CSF from mixed osteoblast marrow cell cultures [Akiyama et al., 2003]. The lack of m-CSF resulted in selective increases in Bim, whereas Bid, Bax, and Bcl-xL were not altered. The increase in Bim was the consequence of reduced ubiquitylation and proteasomal degradation. Interestingly bim−/− mice showed mild osteopetrosis despite an increase in the number of osteoclasts. A role for Fas in osteoclast apoptosis was shown by studies in mice [Wu et al., 2003]. Lpr mice, which have a defective Fas gene [Adachi et al., 1993; Wu et al., 1993], had an increase in osteoclasts and characteristics of elevated resorption. GLD mice, which have inactive Fas ligand [Wu et al., 1993] also had a marked increase in osteoclasts and suggestion of increased resorption. The Fas receptor was present in normal mouse, human and avian osteoclasts and in the RAW 264.7 osteoclast precursor cell line [Wu et al., 2003].

THERAPEUTICALLY EFFECTIVE ANTIRESORPTIVE AGENTS THAT ELICIT OSTEOCLAST APOPTOSIS

Bisphosphonates

Bisphosphonates were shown to have apoptotic effects on osteoclasts in studies published in the mid-1990s. Hughes et al. [1995] devised a model to study osteoclast apoptosis. Bone marrow was obtained from 4- to 6-week old mice. Adherent and non-adherent cells were examined for characteristics of apoptosis (chromatin condensation, nuclear fragmentation, stronger TRAP expression, acridine orange fluorescence, TUNEL staining) or necrosis (swelling, less intense cytoplasmic, and nuclear staining). Three bisphosphonates, pamidronate, clodronate, and risedronate were tested, and all were found to elicit apoptosis, but not necrosis. Risedronate had the greatest effect of the three agents. Culture conditions affected the response to all three agents, with a greater apoptotic effect when the cells were cultured on plastic rather than dentine. Apoptotic effects were also seen with in vivo treatment [Hughes et al., 1995]. Apoptotic cells were found in the proximal tibial metaphysis of bisphosphonate-treated mice and in calvaria of mice treated with bisphosphonate and interleukin-1, which was given to stimulate osteoclastogenesis. Selander et al. [1996b] used isolated osteoclasts from long bones of 1- to 2-day old rat pups and allowed them to attach to slices of cortical bone that were either untreated or treated with pamidronate or clodronate, or exposed on coverslips. Cells were stained for tartrate-resistant acid phosphatase (TRAP) and with the nuclear dye Hoechst 33258, or examined by TEM, or TUNEL stained, with the criteria of apoptosis being fragmented nuclei and chromosomes and shrunken, vacuolized cytoplasm. Apoptosis in cells on the bone slices was apparent at 6 h and increased progressively at time points up to 24 h. Clodronate was the more effective agent. Macrophages and monocyte-macrophage precursor RAW 264.7 cells were not affected, although in later studies, RAW 264.7 cells have been shown to undergo apoptosis in response to antiresorptive agents [Saintier et al., 2006]. Partial inhibition of the apoptotic effects was elicited with a low concentration (1 nM) of the PKC inhibitor staurosporine (which is often used at higher concentrations to induce apoptosis). Inhibition was also elicited by homocysteine, which is metabolized to 3-deazaadenosylhomocysteine, which can interfere with DNA methylation and prevent apoptosis [Endresen et al., 1993]. It can also elevate cyclic AMP [Prytz et al., 1991], an interesting observation in view of the antiapoptotic effects of calcitonin discussed in a later section.

A detailed time course of the process of bisphosphonate-induced apoptosis in vivo was described for disodium dihydrogen (cycloheptylamino)-methylene-1,1-bisphosphonate in male rats [Ito et al., 1999]. The sequence of events was followed by light and electron microscopy at 0 h, 6 h, 1 day, 2 days, and 3 days and 1 week. Detachment from the cell surface was apparent after 1 day. Chromatin condensation was seen at 2 and 3 days. At day 3, phagocytosis by macrophages was noted, and apoptotic osteoclasts were seen in the spleen and blood vessels. At the ultrastructural level, degradation of the Golgi apparatus was apparent at early stages of the apoptotic process.

Research on the mechanism of the effects of bisphosphonates to elicit apoptosis has revealed the involvement of different biochemical processes for different classes of bisphosphonates. The amino bisphosphonates were shown to act through the mevalonate pathway, preventing the formation of isoprenyl groups critical for the activation of small GTP binding proteins, such as Ras, an intermediate in stimulation of Erk signaling, cytoskeletal function, and other processes [Luckman et al., 1998a,b; Fisher et al., 1999, 2000; Reszka et al., 1999; Rogers et al., 2000]. The non-aminobisphosphonate clodronate, on the other hand, did not have this effect. Activation of small G proteins in J74 macrophages was inhibited by alendronate, risedronate, ibandronate, but not by clodronate [Luckman et al., 1998b]. The apoptotic effect of the aminobisphosphonates, but not the non-aminobisphosphonates, was antagonized by geranylgeraniol which bypassed the step at which the aminobisphosphonates blocked the mevalonate pathway [Reszka et al., 1999]. Clodronate was found to inhibit by an apparently unique mechanism of being converted to an ATP analog that inhibited ATP/ADP exchange in the mitochondrial membrane and resulted in mitochondrial function being impaired [Lehenkari et al., 2002]. A recent study has found that aminobisphosphonates can indirectly result in the generation of an ATP analog with the same

deleterious effects on mitochondrial function and promotion of apoptosis, and that this occurs through accumulation of isopentenyl diphosphate (IPP) due to the downstream block in the pathway [Monkkonen et al., 2006]. The relative efficacies of several bisphosphonates in production of the metabolite in J774 cells were zoledronate > risedronate > ibandronate > alendronate > clodronate = 0. An abstract presented at the 2006 ASBMR meeting [Fisher, 2006] supported the possibility that high dose bisphosphonate could elicit osteoclast apoptosis by a mechanism independent of the mevalonate pathway. In the study, this was shown with high dose intermittent ibandronate and zoledronate.

Another important question being addressed is whether apoptosis is the sole mechanism by which bisphosphonates inhibit resorption. Results from studies using rabbit osteoclasts cultured on bovine bone show that the dose-response curves for osteoclast survival and bone resorption assessed by CTx are not superimposable, with significant CTx release elicited by lower bisphosphonate concentrations than decreases in osteoclast number [Halasy-Nagy et al., 2001]. Also, although the caspase inhibitor Z-VAD reversed the effects of the bisphosphonates on osteoclast number, it did not prevent the inhibition of resorption by the aminobisphosphonates, although it did prevent that of the non-aminobisphosphonates. It is interesting that in the case of another inhibitor of the mevalonate pathway, mevastatin, its effect to disrupt actin rings and decrease pit formation by osteoclasts generated from marrow cultures were not prevented by the caspase inhibitor Z-VAD [Woo et al., 2000].

It is noteworthy that agents may have opposite effects on survival of osteoclasts and osteoblasts. Bisphosphonates have been found to increase osteoblast survival [Plotkin et al., 2006], although they can also induce osteoclast apoptosis [Mackie et al., 2001]. It conceivable that there will be distinct mechanisms for the effects on the two cell types, since a recent study has shown that bisphosphonates with diverse structures can all promote osteoblast survival, but that some of the agents elicited osteoclast apoptosis whereas others were inactive [Plotkin et al., 2006].

The question of whether there can be too much apoptosis, whether excessive or prolonged exposure can overcome normal regulatory mechanisms and have irreversible deleterious effects on bone is worth considering, in view of the description by Odvina et al. [2005] of bisphosphonate-treated patients in which all cellularity was lost in iliac crest biopsies of bone. Some of the patients were receiving combinations of bisphosphonate and estrogen, or bisphosphonate and glucocorticoids, although others were being treated with only bisphosphonate. All received calcium and vitamin D. It would seem that increased apoptosis would not be a sufficient mechanism to explain the loss of cellularity, since cells would continue to be generated and this process would also have to be prevented.

Estrogen and SERMS

Estrogens and SERMS have also been found to induce osteoclast apoptosis. This was reported in 1996 in a study by Hughes et al. [1996], using the morphological criteria described above for their earlier report on bisphosphonates [Hughes et al., 1995]. The 17β-estradiol, at 100 pM, increased the percentage of apoptotic osteoclasts after 24 h treatment with the hormone in cultures of bone marrow cells isolated from tibiae and femora of 4- to 6-week old mice. Approximately 10% of the osteoclasts were apoptotic with this and higher concentrations, a 1.7- to 3.2-fold increase over what was seen in controls. The 17β-estradiol also increased apoptosis in TRAP-positive mononuclear osteoclast precursor cells. Tamoxifen also elicited osteoclast apoptosis in the osteoclasts and precursors. The effects were shown to be mediated through TGF-beta, in that they were prevented by a TGF-β pan-specific antibody. In vivo, 17β-estradiol increased the percentages of apoptotic osteoclasts after 1 or 3 weeks treatment of ovariectomized and sham operated mice as indicated by nuclear fragmentation and condensation, intense TRAP staining and acridine orange and TUNEL staining. The authors report the similarity of their findings to those in an earlier study in which estrogen treatment led to disintegration of osteoclasts, although the earlier study did not identify the effect as apoptosis [Liu and Howard, 1991]. In other studies, estrogen and raloxifene were shown to increase TGFβ3 expression in femurs of ovariectomized mice [Yang et al., 1996]. TGFβ stimulates expression of the RANKL decoy

receptor, osteoprotegerin [Murakami et al., 1998; Takai et al., 1998; Thirunavukkarasu et al., 2001], which could provide a mechanism for the effect of estrogen to impair osteoclast survival.

Other studies confirm the effects of estradiol to induce apoptosis. Kameda et al. [1997] demonstrated apoptotic effects in purified mammalian osteoclasts, and found that the effects were antagonized by the SERMs tamoxifen and the pure antagonist ICI 164,384. In the precursor cell line FLG 29.1, estradiol effects to induce apoptosis were demonstrated using time-lapse videomicroscopy, electron microscopy and caspase-3 assay [Zecchi-Orlandini et al., 1999]. A recent study in which the RAW 264.7 cell line was used showed that treatment with 10 nM 17β-estradiol for 5 days enhanced activity of caspases 3, 8, and 9, and increased the Bax/Bcl2 ratio, effects that were antagonized by the SERM ICI 182,780 [Saintier et al., 2006]. Cell differentiation, cell attachment and β3 integrin expression were inhibited by the estradiol, and the cells underwent nuclear condensation. A novel approach showing an effect of estrogen to induce osteoclast apoptosis was the use of a decoy oligonucleotide that mimicked a region of distal promoter C of the ERα gene. This construct induced apoptosis in osteoclasts in an estrogen dependent manner and increased caspase 3 and Fas receptor [Piva et al., 2005]. A difference between two SERMs, raloxifene and ormeloxifene, was found in their abilities to produce apoptosis in bone marrow-derived osteoclasts from Balb/c mice [Narayana Murthy et al., 2006]. The cells were cultured on coverslips and treated for 48 h with each of the SERMS or ethynylestradiol at concentrations of 1, 10, or 1,000 nM, and TUNEL assay used to assess apoptosis. Raloxifene failed to elicit apoptosis, which was elicited by the other two agents in this in vitro model, although raloxifene was more potent in inhibiting osteoclastogenesis. Their in vivo results suggested that raloxifene was somewhat less effective than in other two treatments on bone mineral density of the proximal femur in ovariectomized rats, which could support an association between antiresorptive activity and osteoclast apoptosis.

In the section discussing the bisphosphonates, it was noted that effects of the agents on survival of osteoblasts may be quite different from what occurs in osteoclasts. This is also the case with estrogen [Zallone, 2006].

CALCITONIN: A THERAPEUTICALLY EFFECTIVE ANTIRESORPTIVE AGENT THAT DOES NOT ELICIT OSTEOCLAST APOPTOSIS

Calcitonin inhibits osteoclast activity, however several studies have shown that it does not kill osteoclasts. In contrast, calcitonin prevents their apoptosis, through a PKA pathway [Selander et al., 1996a]. Calcitonin was even able to completely protect osteoclasts from the effects of a nitric oxide releasing compound, a highly effective apoptotic stimulus [Kanaoka et al., 2000]. The effects of calcitonin were mimicked by forskolin and dibutyryl cAMP. The increase in cAMP decreases osteopontin, resulting in cell detachment, which could interfere with resorption. It is interesting that the detachment does not result in apoptosis, since this usually leads to a form of apoptotic cell death known as anoikis [Grossman, 2002]. The results could suggest that the pro-survival signal is able to overcome the deficiencies in survival signaling resulting from lack of attachment. The findings also raise the possibility that osteoclasts, possibly due to their origin from circulating cells in the marrow, might be less susceptible to anoikis. However, studies on effects of osteoclast integrins, described below, suggest that this is not the case. Although the findings on calcitonin point to the fact that apoptosis is not essential for antiresorptive activity, it is interesting to note that calcitonin is less efficacious in the treatment of osteoporosis than bisphosphonates or estrogenic compounds. Thus, apoptosis could result in more effective antiresorptive action.

CALCINEURIN INHIBITORS: AGENTS THAT ELICIT OSTEOCLAST APOPTOSIS BUT ARE NOT USEFUL ANTIRESORPTIVE AGENTS

The calcineurin inhibitors cyclosporine A and tacrolimus have complex and intriguing effects on bone. Because they can block the dephosphorylation and thus the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT) [Clipstone and Crabtree, 1992], they prevent the production of inflammatory cytokines and thus are useful agents for preventing transplant rejection. They also can prevent bone loss in inflammatory conditions, such as rheumatoid arthritis. In vitro, they inhibit the bone resorption elicited in bone organ cultures by a wide range of compounds, including not only the inflammatory

interleukins and prostaglandins, but also parathyroid hormone and calcitriol [Stewart et al., 1986; Klaushofer et al., 1987]. Since all of these ligands can increase RANKL by cells within the culture, these findings are consistent with the observations that the calcineurin inhibitors interfere with RANKL-induced osteoclastogenesis [Ishida et al., 2002; Day et al., 2004; Hirotani et al., 2004; Kim et al., 2005]. The agents can also induce osteoclast apoptosis, which was at least partially prevented by the caspase inhibitor z-VAD [Igarashi et al., 2004], and were more effective when added later in the course of stimulated osteoclastogenesis, suggesting that their effects on apoptosis were more important that their effects on formation of osteoclasts. These effects suggested that the calcineurin inhibitors might be broadly useful antiresorptive agents. However, in vivo studies have found that in non-inflammatory states the calcineurin inhibitors are associated with bone loss in rats and humans [Movsowitz et al., 1988; Thiebaud et al., 1995; Rodino and Shane, 1998]. A study in mice indicates that the sex of the animals can determine whether cyclosporine A protects bone or promotes bone loss [Erben et al., 2003]. The issue is made even more complex by the fact that both anabolic [Tang et al., 2002; Yeo et al., 2006] and antianabolic [McCauley et al., 1992; Fornoni et al., 2001] effects of the calcineurin inhibitors have been described in osteoblastic cells, and enhanced expression of calcineurin or nuclear NFAT in osteoblasts increases osteoblast anabolic activity [Koga et al., 2005; Sun et al., 2005; Winslow et al., 2006].

NEWER ANTIRESORPTIVE AGENTS AND EXPERIMENTAL COMPOUNDS: WHERE DO THEY FIT?

As described above, RANKL interaction with RANK in osteoclasts activates antiapoptotic signaling pathways involving molecules including Src, PI3kinase, Akt, S6kinase, NFκB, and ERKs. The RANK neutralizing antibody denosumab could therefore act at least partially through promoting osteoclast apoptosis. Other antiresorptive agents that interact with these survival pathways include pyrrolopyrimidine Src inhibitors [Recchia et al., 2004]. The cathepsin K inhibitor, beta-cryptoxanthine, is antiresorptive and promotes osteoclast apoptosis [Uchiyama and Yamaguchi, 2006]. Interestingly, cathepsins B, L, and D have been found to promote apoptosis through effects to disrupt mitochondria or activate proapoptotic Bcl family members [Chwieralski et al., 2006], and therefore antagonists of those cathepsins would be expected to be antiapoptotic.

Integrin antagonists could also represent apoptotic antiresorptive agents. Peptide and non-peptide integrin antagonists can prevent bone resorption [Engleman et al., 1997; Lark et al., 1999; Lark et al., 2001; Hutchinson et al., 2003; Wu et al., 2006]. Integrin attachment is a pro-survival event, and activates survival pathways [Cordes, 2006]. An antisense oligonucleotide to the αv integrin subunit dramatically increase the number of osteoclasts with TUNEL-positive nuclei [Villanova et al., 1999], and unoccupied αvβ3 integrin promoted preosteoclast apoptosis [Zhao et al., 2005] as shown by an increase in staining with annexin V, a marker of apoptosis that detects disrupted cell membranes by binding to externalized phosphatidylserine. Paradoxically, the absence of the beta 3 increased survival [Zhao et al., 2005].

Compounds acting by other mechanisms have been reported to elicit osteoclast apoptosis and have been proposed as potential therapeutic agents or representatives of a class of agents that could be developed as therapies. These include V-ATPase inhibitors [Okahashi et al., 1997], the tyrosine kinase inhibitor imatinib mesylate [El Hajj Dib et al., 2006], brefeldin D [Niwa et al., 2001], and a decoy olognucleotide targeting NFκB [Penolazzi et al., 2006] and reveromycin, an antibiotic that inhibits an amino acid transferase [Woo et al., 2006]. As with all therapeutic agents, tissue selectivity, either through targeting or mechanism, is an important factor. As an unusual example, reveromycin selectively induced apoptosis in osteoclasts due to the polarity of the molecule and the requirement of an acid pH such as is present in the environment of the mature active osteoclast to effect permeability [Woo et al., 2006].

SUMMARY

In conclusion, osteoclast apoptosis can be an effective mechanism for antiresorptive agents to elicit their effect, although other mechanisms can lead to inhibition of resorption, and effects on other cells or other actions could counter the apoptotic effects on osteoclasts. It is not clear

whether extensive or prolonged osteoclast apoptosis, in the absence of other inhibitory effects, is harmful to the skeleton. Further examination of this question is warranted, especially in view of current interest in the role of antiresorptive agents in osteonecrosis of the jaw. The finding that activation of caspases can promote cell differentiation as well as apopotosis is most intriguing. It is worth considering as a basis for the disparate effects of an agent on different cell, types or even in the same cell, and as an explanation for why inhibition of osteoclast apoptosis does not necessarily mean that an agent will have a net effect to prevent bone loss in in vivo.

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