Osteoblast Programmed Cell Death (Apoptosis): Modulation by Growth Factors and Cytokines

ROBERT L. JILKA, ROBERT S. WEINSTEIN, TERESITA BELLIDO, A. MICHAEL PARFITT, and STAVROS C. MANOLAGAS

ABSTRACT

Once osteoblasts have completed their bone-forming function, they are either entrapped in bone matrix and become osteocytes or remain on the surface as lining cells. Nonetheless, 50–70% of the osteoblasts initially present at the remodeling site cannot be accounted for after enumeration of lining cells and osteocytes. We hypothesized that the missing osteoblasts die by apoptosis and that growth factors and cytokines produced in the bone microenvironment influence this process. We report that murine osteoblastic MC3T3-E1 cells underwent apoptosis following removal of serum, or addition of tumor necrosis factor (TNF), as indicated by terminal deoxynucleotidyl transferase–mediated dUTP-nick end labeling and DNA fragmentation studies. Transforming growth factor-β and interleukin-6 (IL-6)–type cytokines had antiapoptotic effects because they were able to counteract the effect of serum starvation or TNF. In addition, anti-Fas antibody stimulated apoptosis of human osteoblastic MG-63 cells and IL-6–type cytokines prevented these changes. The induction of apoptosis in MG-63 cells was associated with an increase in the ratio of the proapoptotic protein bax to the antiapoptotic protein bcl-2, and oncostatin M prevented this change. Examination of undecalcified sections of murine cancellous bone revealed the presence of apoptotic cells, identified as osteoblasts by their proximity to osteoid seams and their juxtaposition to cuboidal osteoblasts. Assuming an osteoblast life span of 300 h and a prevalence of apoptosis of 0.6%, we calculated that the fraction that undergo this process in vivo can indeed account for the missing osteoblasts. These findings establish that osteoblasts undergo apoptosis and strongly suggest that the process can be modulated by growth factors and cytokines produced in the bone microenvironment. (J Bone Miner Res 1998;13:793–802)

INTRODUCTION

REGENERATION, A PROCESS common to several tissues, is also essential for the homeostasis of the skeleton and the preservation of its anatomical integrity. Thus, the adult skeleton is continuously remodeled, through the replacement of old bone with new, by a team of juxtaposed osteoclasts and osteoblasts, comprising the so-called basic multicellular unit (BMU).1 During the last few years, it has become evident that orderly genesis of osteoclasts and osteoblasts from their respective progenitors in the bone marrow is an essential determinant of the number of either cell type in the BMU, and therefore critical for the maintenance of bone homeostasis. Indeed, an overproduction of osteoclasts relative to the need for remodeling and an under supply of osteoblasts relative to the need for cavity repair are fundamental pathophysiologic changes in postmenopausal and age-related osteopenia, respectively.2,3

Apoptosis, or programmed cell death, is an important determinant of the life span of cells in regenerating tissues.4,5 Cells undergoing apoptosis are recognized by condensation of chromatin, the degradation of DNA into oligonucleosome-sized fragments, and the formation of plasma and nuclear membrane blebs. Eventually the cell breaks apart to form so-called apoptotic bodies. This process was recently demonstrated in osteoclasts6,7 and, in concert with the rate of osteoclast development, is thought to play a role in the pathophysiology of postmenopausal
In the case of osteoblasts, it was recently shown that osteoblastic cell lines undergo apoptosis in vitro, but few if any apoptotic osteoblasts were detected by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) in osteophytes or in fetal bone. Nonetheless, it is widely accepted that once osteoblasts have completed their bone-forming function, they face one of two fates. They are either entrapped in the matrix and become osteocytes or remain on the surface of the newly formed bone and become lining cells. Analysis of available data from histomorphometric examinations of human bone has revealed that 50–70% of the osteoblasts initially present at the remodeling site cannot be accounted for after enumeration of lining cells and osteocytes. Based on this evidence, we have hypothesized that the “missing” osteoblasts must have died, possibly by apoptosis. Furthermore, we have reasoned that if apoptosis does occur in osteoblasts or their progenitors, by analogy with the case of osteoclasts, the incidence of apoptosis in concert with osteoblastogenesis would determine the number of osteoblasts in the BMU and thereby the rate of bone formation.

It is widely accepted that growth factors and cytokines produced in the bone microenvironment are potent modulators of the rate of both osteoclast and osteoblast development. Based on this, and evidence that such agents influence the incidence of cell apoptosis in other tissues, and in the case of transforming growth factor-β (TGF-β) osteoclast apoptosis, we reasoned further that growth factors and cytokines that influence osteoblast replication and differentiation, such as interleukin-6 (IL-6)–type cytokines and TGF-β, may also influence osteoblast apoptosis.

MATERIALS AND METHODS

Materials

Recombinant murine or human tumor necrosis factor (TNF), murine TGF-β, murine leukemia inhibitory factor (LIF), human oncostatin M (OSM), and human interferon-γ (IFN-γ) were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Monoclonal murine anti-human Fas antibody was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Synthetic parathyroid hormone (1–34) was from Peninsula Laboratories (Belmont, CA, U.S.A.), and 1,25-dihydroxyvitamin D₃ was a gift from Hoffman-LaRoche (Nutley, NJ, U.S.A.).

Detection of apoptosis in cultured cells

The murine calvaria-derived osteoblastic cell line MC3T3-E1 and the human osteosarcoma cell line MG-63 were cultured in phenol red-free minimal essential medium (MEM; GIBCO-BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% FBS (Hyclone Corp., Logan, UT, U.S.A.). Cells exhibiting DNA fragmentation, and therefore undergoing apoptosis, were visualized using TUNEL with reagents obtained from Oncor (Gaithersburg, MD, U.S.A.) or from Oncogene (Cambridge, MA, U.S.A.). Cells were fixed with 2% paraformaldehyde, permeabilized with 1% Triton X-100 in 0.1 sodium citrate, and then incubated with terminal transferase and fluorescein isothiocyanate (FITC)-labeled dUTP. Cells with FITC-labeled DNA were visualized by fluorescence microscopy.

To demonstrate formation of oligonucleosome-sized DNA fragments during apoptosis, cells were removed from the tissue culture dish and lysed with 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 20 μg/ml RNase A, and 0.5% sodium dodecyl sulfate (SDS) for 1 h at 37°C. Proteinase K was added (100 μg/ml) and the lysate incubated for an additional 3 h at 50°C. DNA was then extracted with phenol saturated with 100 mM Tris-buffer, pH 7.4, and precipitated with ethanol. After washing with 75% ethanol, reprecipitation, and redigestion with RNase A, DNA was analyzed on a 1% agarose gel containing ethidium bromide.

Quantitation of apoptotic cells

Apoptotic cells were quantitated after combining nonadherent cells with adherent cells released from the culture dish using trypsin-EDTA. Cells were resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) prior to analysis. For determination of dead cells by dye exclusion, 0.4% Trypan Blue was added, and the percentage of cells exhibiting both nuclear and cytoplasmic trypan blue staining was determined using a hemocytometer. A minimum of 100 cells were counted. For determination of apoptotic cells using flow cytometry after TUNEL labeling, cells were fixed with 2% paraformaldehyde, rinsed with PBS, and the TUNEL reaction was then carried out as described above using FITC-dUTP. The labeled cells were then subjected to flow cytometry using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). The percentage of cells that were labeled, and thus undergoing apoptosis, was determined after electronic subtraction of signal due to background fluorescence, which was determined using cells incubated with FITC-labeled dUTP, but without terminal deoxynucleotidyl transferase. A minimum of 5000 cells were analyzed.

Detection of Bcl-2 and Bax by Western blot analysis

MG-63 cells (adherent and nonadherent) were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, 0.14 U/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 and centrifuged at 14,000 rpm for 10 minutes to remove insoluble material. Lysates were dissolved in SDS electrophoresis buffer and proteins were separated on SDS-polyacrylamide gels and subsequently electrotransferred to polyvinylidene difluoride membranes. After blocking with PBS containing 0.1% Tween 20 and 5% nonfat dry milk, membranes were incubated overnight at 4°C with an antibody to either Bcl-2, Bax, (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), or actin (Sigma, St. Louis, MO, U.S.A.), and the bound antibodies were detected with the corresponding secondary antibody conjugated with horse radish peroxidase. Blots were developed by enhanced chemiluminescence using reagents from New England Nuclear (Boston, MA, U.S.A.). Quantitation of the intensity of the bands in the autoradiograms was per-
formed using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Measurements were made after appropriate exposure of the film to assure that the pixel volume was in the linear range of detection, based on a previously established calibration curve.

Detection of Fas antigen expression

MG-63 cells cultured on glass slides were fixed with 4% paraformaldehyde in PBS for 5 minutes at 4°C. After rinsing and blocking with 100 μg/ml murine immunoglobulin G (IgG) in PBS, cells were incubated for 30 minutes at 4°C with 1 μg/ml of murine anti-human Fas antibody. After rinsing in PBS, bound antibody was visualized by fluorescence microscopy using FITC-labeled goat anti-mouse Ig (Becton Dickinson, San Jose, CA, U.S.A.).

Detection of apoptotic cells in cancellous bone

Murine femurs were fixed in Millonig’s buffered formalin, dehydrated, and embedded in methyl methacrylate as previously described.3 Paired pairs of undecalcified 3- to 4-μm-thick longitudinal sections were cut, mounted on precoated glass slides (SectionLock, Polysciences, Inc., Warrington, PA, U.S.A.), and then deplasticized. Bone sections were analyzed for cells exhibiting DNA fragmentation, and therefore undergoing apoptosis, using the TUNEL procedure using reagents from Oncor. After digestion with 100 μg/ml proteinase K for 15 minutes at room temperature, endogenous peroxidase was quenched with 3% H2O2. After placement in equilibration buffer, terminal deoxynucleotidyl transferase and digoxigenin-labeled dUTP were added and sections were incubated for 1 h at 37°C. After rinsing in stop-wash buffer, TUNEL signal was detected with peroxidase labeled antidigoxigenin antibody and developed with diaminobenzidine.16 Sections were counterstained with methyl green. Just weaned rat mammary tissue, supplied by Oncor, was used as a positive control, and negative controls were made by omitting the transferase from the reaction mixture from one of each pair of bone sections, or from the section of mammary tissue.

Statistics

Data were analyzed using SigmaStat (Jandel Scientific, San Rafael, CA, U.S.A.). Statistically significant differences in the number of apoptotic cells among various treatment groups was determined using either Chi-square analysis or analysis of variance.

RESULTS

To test our hypothesis that osteoblasts undergo apoptosis, we first searched for the phenomenon in an established murine osteoblastic cell line, MC3T3-E1. A small portion of MC3T3-E1 cells underwent apoptosis when maintained in the presence of 10% FBS as indicated by TUNEL labeling of nuclei with FITC-dUTP (Fig. 1A). However, the number of apoptotic cells was dramatically increased following removal of serum, a well-established strategy for inducing apoptosis14 (Fig. 1B). Addition of TNF, another established inducer of apoptosis,19 stimulated apoptosis of MC3T3-E1 cells (Fig. 1C). When the TUNEL reaction in these experiments was carried out in the absence of transferase, cells exhibiting fluorescence were scarce (Fig. 1D), confirming the specificity of the assay.

The occurrence of apoptosis, as opposed to necrosis, in MC3T3-E1 cells was confirmed by the demonstration of 180 bp DNA fragments, and multimers thereof, in cell extracts (Fig. 2). The demonstration of such fragments is a sine qua non feature of apoptosis and results from intranucleosomal DNA degradation by enzymes induced during programmed cell death.18

We quantitated the number of apoptotic cells using either flow cytometry, following TUNEL-labeling, or trypan blue staining. The percentage of cells labeled by the TUNEL method in these experiments was determined by electronically subtracting the fluorescence signal in cells not exposed to transferase during TUNEL labeling. The number of cells exhibiting a fluorescence signal outside this boundary were considered apoptotic. Trypan blue staining is based on the principle that dying cells lose membrane integrity, thereby allowing entry of the dye into their interior. A summary of the quantitative analysis of apoptotic MC3T3-E1 cells, using either flow cytometry or trypan blue staining from three different experiments, is provided in Table 1. Although the absolute number of dead cells detected by trypan blue staining and the number of TUNEL-labeled cells differed, comparable results were obtained using both methods. Therefore, apoptotic osteoblasts could be reliably quantitated by either method.

Using these techniques, we examined whether TGF-β could induce apoptosis, or whether it would influence apoptosis induced by serum removal or the addition of TNF. Representative flow cytometry histograms displaying the effect of TGF-β on the apoptosis induced by serum removal are shown in Fig. 3. MC3T3-E1 cells cultured for 24 h in the presence of 10% FBS, consistent with the results of experiments shown in Fig. 1. Addition of 0.5 nM TGF-β suppressed the increased fluorescence labeling induced by the removal of serum. Similarly, TGF-β inhibited apoptosis induced by the addition of TNF. TGF-β by itself, however, had no influence on apoptosis (Table 1).

Cytokines that utilize the gp130 signal transducer, such as IL-6 and LIF, are potent antiapoptotic agents in other cell types and inducers of osteoblast differentiation.19–21 We therefore wished to examine their influence on osteoblast apoptosis. Addition of LIF to MC3T3-E1 cells, which express functional receptors for LIF,22 inhibited apoptosis stimulated by the removal of serum or by the addition of TNF (Table 1, experiment 3). Addition of 0.5 nM sIL-6R to cells maintained in the presence of 10% FBS had no effect on apoptosis. In studies not shown here, we also determined that parathyroid hormone and 1,25-dihydroxyvitamin D3 had no influ-
ence on apoptosis either under basal conditions or following induction of the phenomenon by removal of serum or by addition of TNF.

To demonstrate that the antiapoptotic properties of TGF-β and IL-6–type cytokines in osteoblasts were not limited to murine cells, we extended these experiments using the human osteosarcoma osteoblast-like cell line MG-63. Unlike MC3T3-E1 cells, MG-63 cells do not express the LIF receptor β but do express the type II oncostatin M receptor. In contrast to MC3T3-E1 cells, MG-63 cell apoptosis was not influenced by the removal of serum or the addition of TNF (Table 2, experiment 1). However, addition of anti-Fas antibody stimulated apoptosis, and this effect was more pronounced following treatment of the cells with IFN-γ, which is known to enhance expression of Fas antigen. Activation of gp130 signaling by OSM, or by IL-6 + sIL-6R, suppressed apoptosis in MG-63 cells (Table 2, experiment 2). However, although TGF-β exerted antiapoptotic effects in some experiments, this effect was not reproducible (data not shown). The antiapoptotic effects of the IL-6–type cytokines was not due to inhibition of Fas expression on MG-63 cells, as indicated by the lack of effect of these factors on staining of Fas with murine anti-Fas antibody and FITC-labeled goat anti-mouse Ig (data not shown). Stimulation of apoptosis by Fas signaling is often mediated by changes in the synthesis of the bcl family of proteins that are involved in the regulation of apoptosis. Consistent with this, we found that the increase in apoptotic MG-63 cells upon exposure to IFN-γ + anti-Fas was associated with a decrease in the level of the antiapoptotic protein Bcl-2 and an increase in the level of Bax, a proapoptotic protein (Fig. 4). Simultaneous addition of OSM prevented these changes.

Finally, in an attempt to demonstrate whether the evi-
dence from the in vitro experiments described above was relevant to the in vivo situation, we searched for cells exhibiting apoptotic features in nondecalcified sections of cancellous murine bone using the TUNEL method. Cells exhibiting TUNEL labeling were observed on the surface of the secondary spongiosa of the distal femur of 3-month-old mice SAMR1 mice, a substrain of AKR/J mice (Figs. 5A and 5B). TUNEL labeling of cells in the bone sections was specific, as demonstrated by the absence of TUNEL label in adjacent cells, and a lack of labeling when transferase was omitted from the reaction mixture (Fig. 5C). The apoptotic cell shown in Fig. 5B is next to cells that appear to be in the

**FIG. 2.** Apoptosis in MC3T3-E1 osteoblastic cells detected by DNA fragmentation. MC3T3-E1 cells were cultured in 75 cm² flasks in MEM with 10% FBS, or with 0.5% BSA (“serum-free”), for 24 or 48 h (left panel); or in the presence of MEM with 10% FBS, or with 0.5% BSA (“SF”), or 1 nM TNF, for 24 h (right panel). DNA was extracted and 5–10 μg was analyzed by electrophoresis on a 1% agarose gel. DNA was visualized with ethidium bromide.

**TABLE 1. REGULATION OF APOPTOSIS IN MURINE OSTEOBLASTIC MC3T3-E1 CELLS**

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<td>10% FBS + TNF + IL-6 + sIL-6R</td>
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Experiment 1—Cells were cultured for 16 h in MEM containing 10% FBS in the absence or the presence of 0.5 nM TGF-β. Then, the medium was replaced with 10% FBS, or with 0.5% BSA in MEM (without or with 0.5 nM TGF-β). Apoptosis was quantified 24 h later by flow cytometry after TUNEL labeling, or by trypan blue staining. Experiment 2—Cells were cultured in the absence or presence of 0.5 nM TGF-β as in experiment 1. Then the medium was replaced with 10% FBS (without or with 0.5 nM TGF-β), or with 10% FBS containing TNF, without or with 0.5 nM TGF-β. Apoptosis was quantified 24 h later as in experiment 1. Experiment 3—Cells were cultured for 16 h in MEM with 10% FBS in the absence or the presence of 1 nM LIF, or the combination of 1 nM IL-6 and 15 nM IL-6sR. Then, the medium was replaced with 10% FBS, or 0.5% BSA in MEM, or 10% FBS with 1 nM TNF, and maintained for an additional 24 h in the absence or presence of the cytokines used in the pretreatment phase. Apoptosis was quantified by trypan blue staining. Data from experiments 1 and 2 were from single determinations and analyzed by Chi-square test; data from experiment 3 were from triplicate cultures and were analyzed by analysis of variance. Similar results were obtained in at least one additional experiment. *p < 0.05 versus cells maintained in the presence of 10% FBS; †p < 0.05 versus cells cultured in 0.5% BSA, or in the presence of TNF.
process of becoming trapped in a mineralized matrix. The TUNEL-labeled cells in Fig. 5 are also in close proximity to cells that were identified as osteoblasts based on their cuboidal morphology and the apical location of their nucleus as well as the juxtaposition of these cells to osteoid. Based on these observations, we identified these TUNEL-labeled cells as osteoblasts. Upon examination of bones from three animals, we found that 6 out of 1000 osteoblasts exhibited TUNEL labeling similar to that shown in Fig. 5. Apoptotic cells were also observed in cells present in the bone marrow space, but their identity was not determined.

DISCUSSION

The results of the experiments described in this paper demonstrate that apoptosis occurs in both murine and human osteoblastic cell lines and that it is influenced by factors produced in the bone marrow microenvironment. In addition, they reveal the occurrence of apoptotic osteoblasts in murine cancellous bone.

Consistent with our findings, a stimulating effect of TNF on apoptosis of MC3T3-E1 cells and murine osteoblastic cells from neonatal calvaria has been reported previously.\(^{(9,10)}\) Interestingly, however, in the present report we observed that MG-63 cells failed to undergo apoptosis in response to TNF, even though they exhibit responsiveness to this cytokine when other parameters have been tested.\(^{(25)}\) However, apoptosis could be stimulated in these cells following treatment with IFN-\(\gamma\), an inducer of Fas antigen expression, and subsequent activation with anti-Fas antibody. Fas-activated apoptosis is mediated by several signaling pathways, whereas TNF both stimulates the activation of apoptosis and the production of factors that inhibit apoptosis via NF-\(\kappa\)B.\(^{(26,27)}\) Thus, the different apoptotic response of MC3T3-E1 cells and MG-63 cells to TNF may be explained by the presence of the latter pathway in MG-63 cells, but not in MC3T3-E1 cells.

The antiapoptotic effects of IL-6-type cytokines and TGF-\(\beta\) on osteoblastic cells noted here are similar to those reported for other cell types.\(^{(19,28,29)}\) Induction of apoptosis by anti-Fas in our studies was associated with an increase in the ratio of Bax to Bcl-2. This finding is in line with evidence that an increase in this ratio favors formation of bax homodimers which can trigger cell death.\(^{(24)}\) The ability of OSM to prevent both Fas-induced apoptosis and an increase in the Bax to Bcl-2 ratio suggests that gp130 signaling interferes with early events responsible for the induction of apoptosis in osteoblasts. In support of this contention, we have recently found that an increase in the synthesis of p21\(^{\text{WAF1,CIP1}}\), a protein involved in the regulation of the cell cycle, mediates the antiapoptotic effects of IL-6-type cytokines in MG-63 cells.\(^{(30)}\)

As opposed to the antiapoptotic effect of TGF-\(\beta\) noted here, it has been reported that this factor promotes apoptosis in other cells.\(^{(31)}\) Of particular interest is the recent demonstration that TGF-\(\beta\) mediates the ability of 17\(\beta\)-estradiol to stimulate osteoclast apoptosis.\(^{(18)}\) Thus, the increased bone remodeling caused by estrogen deficiency
might be due not only to increased osteoclastogenesis and osteoblastogenesis, resulting in part from increased production and/or sensitivity to IL-6–type cytokines, but also to an increase in the life span of these cells.

Because of its transient nature, apoptosis can be difficult to detect and quantitate in vivo unless the tissue of interest is undergoing rapid cell turnover, such as regenerating lung and liver following injury. To search for apoptotic osteoblasts, we employed sections of the secondary spongiosa of the femur, a site that undergoes remodeling as opposed to modeling, and we concentrated on fields containing matrix synthesizing osteoblasts so as to optimize our chances of visualizing some of these cells as they were undergoing apoptosis. Further, we employed the TUNEL staining method because it has been shown to be a useful technique for quantitation of apoptosis in tissue sections and is especially useful for those tissues for which morphological identification of apoptotic cells is difficult. Finally, we used plastic-embedded undecalcified bone sections because this technique avoids exposure of the tissue to hot liquid paraffin, which has been reported to artefactually increase the number of apparent apoptotic cells.

Apoptotic cells that could be clearly identified as osteoblasts were observed in the sections and they occurred at a frequency of ~0.6%. The use of undermineralized bone sections was crucial in the detection of apoptotic osteoblasts because they were found lining unmineralized bone matrix, or osteoid, deposited over mineralized bone. To deduce the biologic relevance of the phenomenon, we used previous measures of bone formation (obtained using dynamic histomorphometry) at the murine secondary spongiosa and calculated the mean active life span of osteoblasts at this site by dividing the wall width by the mineral appositional rate. From these calculations, we estimated that the mean active life span of osteoblasts at this site is about 12 days or 288 h. In a steady state, the fraction of cells at a particular stage is the same as the corresponding fraction of time spent in that stage. Assuming the osteoblast life span to be ~300 h and the prevalence of apoptotic osteoblasts cells to be 0.006, as determined in the present study, the following relationship will hold:

\[ t_{\text{Ap}}/300 = 0.006/f_{\text{Ap}} \]

where \( t_{\text{Ap}} \) is the duration (in hours) of the DNA fragmentation phase of apoptosis that is detected by TUNEL and \( f_{\text{Ap}} \) is the fraction of osteoblasts that undergoes apoptosis. Based on a value of \( t_{\text{Ap}} \) in the range of 2–3 h, determined previously for regenerating liver, the corresponding values for \( f_{\text{Ap}} \) are 0.6–0.9. These calculations demonstrate that the prevalence of apoptosis we observed, although apparently low, is consistent with the conclusion drawn from studies of human bone that 50–70% of osteoblasts undergo apoptosis, and that only a minority become osteocytes or lining cells. Furthermore, they suggest that prevention or postponement of apoptosis could correct the deficit in the number of osteoblasts at bone-forming sites which characterizes senile osteoporosis.

As opposed to apoptotic osteoblasts, we did not observe apoptotic osteoclasts in our examination of the remodeling bone of the secondary spongiosa of the 3-month-old mice used in the present studies. However, apoptotic osteoclasts were detected by Boyce and colleagues in the primary spongiosa, a site that undergoes rapid modeling of growing 5-week-old mice. It is possible that our inability to detect apoptotic osteoclasts is due to methodologic differences (use of mineralized bone sections in our studies as opposed to modeling, and we concentrated on fields containing matrix synthesizing osteoblasts so as to optimize our chances of visualizing some of these cells as they were undergoing apoptosis. Further, we employed the TUNEL staining method because it has been shown to be a useful technique for quantitation of apoptosis in tissue sections and is especially useful for those tissues for which morphological identification of apoptotic cells is difficult. Finally, we used plastic-embedded undecalcified bone sections because this technique avoids exposure of the tissue to hot liquid paraffin, which has been reported to artefactually increase the number of apparent apoptotic cells.

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Experiment 1—Cells were cultured for 16 h in MEM containing 10% FBS in the absence or the presence of 0.1 nM IFN-γ. Then, the medium was replaced with 10% FBS or with 0.5% BSA in MEM, or with 10% FBS containing 1 nM TNF, or with 10% FBS containing 10 µg/ml anti-Fas antibody. Cells were cultured for an additional 24 h and apoptosis was quantified by trypan blue staining. Experiment 2—Cells were cultured in 10% FBS in MEM in the absence or the presence of 0.1 nM IFN-γ or with 1 nM OSM, or with the combination of 1 nM IL-6 + 15 nM sIL-6R. Then, the medium was replaced with fresh medium containing 10 µg/ml anti-Fas antibody without or with OSM or IL-6 + sIL-6R. Cells were cultured for an additional 24 h and apoptosis was quantified by flow cytometry after TUNEL labeling or by trypan blue staining.
opposed to demineralized sections used by Boyce) or to a lower prevalence of osteoclast apoptosis in remodeling bone as compared with modeling bone in growing animals. Further studies will be required to distinguish between these possibilities.

Previous work of ours has elucidated that either an increase or a decrease in osteoblastogenesis from progenitors in the bone marrow is responsible for respective changes in the rate of bone formation and bone turnover. Specifically, we have documented that following the loss of sex steroids, osteoblastogenesis and bone formation are increased.\(^2,3^7\). However, osteoblastogenesis and bone formation are decreased in a murine model of low turnover osteopenia and accelerated senescence.\(^3,3^5\) In addition, evidence from the studies of others, as well as ours, points to the possibility that the changes in osteoblastogenesis in these conditions

![Graph showing levels of Bcl-2 and Bax proteins in MG-63 cells](image)

**FIG. 4.** Levels of Bcl-2 and Bax proteins in MG-63 cells. MG-63 cells were cultured for 24 h as described in Table 2 in the absence or the presence of IFN-γ alone or in combination with 10 ng/ml OSM, and subsequently incubated with anti-Fas antibody for an additional 16 h. Cell lysates were obtained from both adherent and nonadherent cells and subjected to Western blot analysis with anti–Bcl-2, anti-Bax, and anti–β-actin antibodies as described in the Materials and Methods (upper panel). Levels of Bcl-2 and Bax, relative to actin, were obtained by quantitating the intensity of the bands in the autoradiographs using a laser densitometer (lower panels). These values were used to calculate the ratio of the band intensities of Bax to Bcl-2 listed in the figure. Essentially identical results were obtained in two additional experiments.

![Microphotographs of apoptotic osteoblasts in murine cancellous bone](image)

**FIG. 5.** Microphotographs of apoptotic osteoblasts in murine cancellous bone. Murine femora were fixed in Millonig's buffered formalin, dehydrated, and embedded in methyl methacrylate. Nondecalcified 3- to 4-μm-thick longitudinal sections were cut, mounted on precoated glass slides, and then deplasticized. The bone sections were analyzed for the presence of apoptotic cells using the TUNEL procedure. Sections were counterstained with methyl green. The secondary spongiosa of the distal femur was inspected for the presence of apoptotic cells. Magnification ×400. (A, B) Positively stained apoptotic osteoblasts (indicated by arrowheads) are in close proximity to osteoblasts (indicated by asterisks). In (B), apoptotic osteoblasts are juxtaposed between two osteoblasts (indicated by arrows) that are becoming encased in bone matrix. (C) No apoptotic cells were observed on the bone surface or in the bone marrow when the TUNEL procedure was performed without the transferase enzyme. Osteoblasts are indicated by asterisks.
may be due to alteration in the production of, and responsiveness to, IL-6–type cytokines and perhaps growth factors such as TGF-β.(21,38,39) The results of the present studies strongly suggest that besides their effects on osteoblastogenesis, IL-6–type cytokines and TGF-β may affect the life span of osteoblastic cells, and thereby the number of osteoblasts in the BMU.

At this point, we can only speculate on the mechanisms responsible for induction of osteoblast apoptosis in vivo. Nevertheless, based on our in vitro findings with osteoblastic cell lines, it is likely that osteoblasts may undergo programmed cell death because of increased sensitivity to apoptosis-inducing agents. Alternatively, osteoblasts may undergo apoptosis as a result of decreased concentrations of antiapoptotic growth factors and cytokines in their immediate vicinity. Apoptosis of osteoblasts might also be enhanced as a result of increased concentrations of (or responsiveness to) factors that stimulate apoptosis such as TNF or Fas ligand. The latter two scenarios are consistent with the contention that the ability of IFN-γ and TNF to inhibit bone formation(40) is due, at least in part, to the induction of osteoblast-programmed cell death either directly (in the case of TNF) or secondary to the induction of Fas and subsequent exposure to Fas ligand in the case of IFN-γ.(26)

In conclusion, the evidence described in this manuscript indicates that osteoblast apoptosis occurs in vitro as well as in remodeling murine cancellous bone in vivo. In the in vivo situation, the phenomenon seems to occur with sufficient frequency to have a significant impact on the number of osteoblasts present at the site of bone formation. Alternations in the timing and extent of osteoblast apoptosis could therefore have a significant impact on the rate of bone formation. The evidence that growth factors such as TGF-β and IL-6–type cytokines prevent osteoblast apoptosis suggests further that osteoblast apoptosis is under the regulatory control of factors produced in the bone microenvironment. Future studies to quantify apoptotic osteoblasts in different disease states will be needed to establish the working hypothesis that, similar to the case of bone resorption, the rate of bone formation is regulated by both the rate of osteoblastogenesis and the rate of apoptosis of osteoblasts.

ACKNOWLEDGMENTS

The authors wish to thank Francis Miller, Jeff Woodliff, and Catherine Smith for excellent technical assistance; and Dr Paula Roberson for advice on statistical analysis of the data. This work was supported by the NIH (PO1-AG13918) and the Department of Veterans’ Affairs.

REFERENCES


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Received in original form July 18, 1997; in revised form November 5, 1997; accepted December 8, 1997.