Journal of Cellular Biochemistry 68:31–49 (1998)

Apoptosis During Bone-Like Tissue Development In Vitro

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Abstract We present evidence of cell death by apoptosis during the development of bone-like tissue formation in vitro. Fetal rat calvaria-derived osteoblasts differentiate in vitro, progressing through three stages of maturation: a proliferation period, a matrix maturation period when growth is downregulated and expression of the bone cell phenotype is induced, and a third mineralization stage marked by the expression of bone-specific genes. Here we show for the first time that cells differentiating to the mature bone cell phenotype undergo programmed cell death and express genes regulating apoptosis. Culture conditions that modify expression of the osteoblast phenotype simultaneously modify the incidence of apoptosis. Cell death by apoptosis is directly demonstrated by visualization of degraded DNA into oligonucleosomal fragments after gel electrophoresis. Bcl-X_L, an inhibitor of apoptosis, and Bax, which can accelerate apoptosis, are expressed at maximal levels 24 h after initial isolation of the cells and again after day 25 in heavily mineralized bone tissue nodules. Bcl-2 is expressed in a reciprocal manner to its related gene product Bcl-X_L with the highest levels observed during the early post-proliferative stages of osteoblast maturation. Expression of p53, c-fos, and the interferon regulatory factors IRF-1 and IRF-2, but not cdc2 or cdk, were also induced in mineralized bone nodules. The upregulation of Msx-2 in association with apoptosis is consistent with its in vivo expression during embryogenesis in areas that will undergo programmed cell death. We propose that cell death by apoptosis is a fundamental component of osteoblast differentiation that contributes to maintaining tissue organization. J. Cell. Biochem. 68:31–49, 1998.

Key words: Bax; Bcl-2; Bcl-X; bone; programmed cell death; p53; c-fos; Msx-2; differentiation; IRF-1; IRF-2; collagenase gene expression

Cell death by apoptosis is recognized as an important component of growth during embryogenesis, organogenesis, and tissue morphogenesis as well as in the maintenance of homeostasis in many adult tissues [Clarke, 1990]. Programmed cell death (PCD) by apoptosis functions prominently in early embryonic development as a mechanism to establish fidelity of tissue morphology [Saunders, 1966]. Examples include the formation of digits [Garcia-Martinez et al., 1993; Lee et al., 1993; Hammar and Mottet, 1971], palate fusion [Pratt and Martin, 1975], and tooth development [Bronckers et al., 1996b]. Apoptosis is also essential for development of proper organ function by eliminating T cells in the regressing thymus [Compton and Cidlowski, 1992; Hoffmann et al., 1994; Duke and Cohen, 1986], supporting clonal deletion of B cells [Nossal, 1983] and deletion of supernumerary neurons in the brain [Oppenheim, 1984]. In normal adult tissues such as skin or intestinal epithelia, which undergo rapid renewal of epithelia [Hopwood and Levison, 1976], apoptosis facilitates remodelling by providing an equal and opposite force to mitosis to regulate population size [Kerr et al., 1972].

Apoptotic cells are recognized by distinguishing morphological features. As the cell enters an initial phase [Earnshaw, 1995], loss of cell junctions and other specialized plasma membrane structures can be observed and collapse of the chromatin into one or a few large clumps against the nuclear envelope is evident. The collapse of the chromatin is frequently accompanied by fragmentation of DNA by an endonuclease into oligonucleosomal-sized fragments that

Contract grant sponsor: NIH; Contract grant numbers: AR39588, AR42262.

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Received 17 April 1997; Accepted 14 August 1997

may be visualized by agarose gel electrophoresis. Biochemical events and molecular markers functionally related to the promotion or prevention of apoptosis have been identified. A family of interleukin-1β converting enzyme proteinases may be involved in the activation of endonuclease(s) responsible for the degradation of DNA [Flaws et al., 1995; Miura et al., 1993]. Several genes have been implicated in regulating a decision between cell survival and cell death. Bcl-2 blocks cell death in thymocytes induced by growth factor withdrawal, γ irradiation, and glucocorticoids, but not death induced by all stimuli [Korsmeyer, 1992a]. A homologue of Bcl-2 heterodimerizes with Bcl-2 and thereby inactivates it, promoting cell death [Oltvai et al., 1993]. An additional homologue of Bcl-2, Bcl-X_L, inhibits cell death as effectively as Bcl-2, but an alternatively spliced form, Bcl-X_S, actually inhibits the ability of Bcl-2 to promote cell survival [Boise et al., 1993]. Studies defining the role of Bcl-X and Bcl-2 in regulating a common pathway of cell death have been carried out largely in T cells [Chao et al., 1995].

A number of cell cycle genes (c-fos, c-myc, c-jun, cdc2) and transcriptional activators (p53, IRF-1, and IRF-2) appear to be upregulated in response to apoptosis-inducing stimuli and have been implicated, therefore, as essential mediators of the process [Lowe et al., 1993; Kastan et al., 1991; Taniguchi et al., 1995; Smeyne et al., 1993; Colotta et al., 1992; Bissonette et al., 1992; Shimizu et al., 1995; Shi et al., 1994; Pandey and Wang, 1995]. IRF-1 inhibits cell growth [Harada et al., 1993; Yamada et al., 1990], and IRF-2 is an antagonistic repressor of IRF-1 [Yamamoto et al., 1994]. Recently, IRF-2 was shown to bind to a histone gene cell cycle regulatory element and modulate transcriptional upregulation at the G_1/S phase transition [Vaughan et al., 1995]. Embryonic fibroblasts from IRF-1 deficient mice are refractory to DNA damage-induced cell cycle arrest [Tanaka et al., 1994] similar to embryonic fibroblasts lacking the tumor suppressor gene p53 [Liebermann et al., 1995; Vogelstein and Kinzler, 1992; Donehower et al., 1992] and the lack of either IRF-1 or p53 alone is sufficient to prevent ras-induced apoptosis [Tanaka et al., 1994]. Recent studies suggest that these two tumor suppressor transcription factors are required for distinct apoptotic pathways in T lymphocytes [Tamura et al., 1995].

While cell death during embryonic limb development and during maintenance of homeostatic growth in many adult tissues has been reported, a role for apoptosis in post-natal bone formation and turnover has only recently been investigated. Programmed cell death of hypertrophic chondrocytes in their terminal differentiation stage is requisite for the progression of endochondral bone formation [Hatori et al., 1995; Roach et al., 1995; Gibson et al., 1995]. Early investigations of the cell kinetics of bone development demonstrated morphological and kinetic changes in osteocytes during osteogenesis throughout life that may be ascribed to apoptosis [Zimmerman, 1992; Jande and Bélanger, 1973; Frost, 1960]. It was suggested that empty osteocyte lacunae identified in human bone specimens derived from patients ranging in age from infancy to 84 years were a consequence of cell death [Frost, 1960]. Other investigators described terminal stages of structural and functional changes in the life cycles of osteocytes that are consistent with morphological modifications associated with cell death by apoptosis [Zimmerman, 1992; Jande and Bélanger, 1973]. Specifically, electron micrographs showed osteocytes with irregularly shaped nuclei containing condensed heterochromatin and interrupted nuclear envelopes consistent with morphological characteristics of apoptotic cells. Apoptosis of bone cells has been detected directly in vivo by in situ methods (transferase-mediate, biotin-dUTP^{mick} end-labeling [TUNEL] assay) in osteocytes [Noble et al., 1997; Bronckers et al., 1996a], in dentin repair [Bronckers et al., 1996b], and in the multinucleated osteoclast which functions in resorption of bone matrix [Boyce, 1996].

Despite the accrual of evidence for involvement of apoptosis in skeletal development and remodelling, the signaling events or factors regulating decisions for apoptosis have not been investigated in bone tissues or isolated cells. To begin addressing fundamental mechanisms associated with apoptosis in osteoblasts, we investigated the incidence of PCD in cultured fetal rat calvarial osteoblasts [Owen et al., 1990], a well-characterized in vitro model of intramembranous bone development. Cell cultures derived from fetal calvaria undergo a temporal expression of genes characterizing stages in development of the osteoblast phenotype. The

cells proliferate and multilayer in discrete focal nodules that develop a bone-like tissue organization. Coincident with cellular multilayering, type I collagen fibrils are deposited in orthogonal layers between the cellular multilayers analogous to the organization of the collagenous extracellular matrix (ECM) synthesized by osteoblasts in vivo [Pockwinse et al., 1992; Lynch et al., 1995]. In intact bone, preosteoblasts develop from mesenchymal precursors on the periosteal surface, divide and differentiate into osteoblasts [Aubin et al., 1993]. In our model system, the preosteoblasts correspond to those cells that are actively proliferating. At this stage of maturation, expression levels are high for genes that influence the outcome of mesenchymal cells en route to the skeletal lineage during early development, e.g., the bone morphogenetic proteins and the homeodomain protein Msx-2 [Tabin, 1995; Hoffmann et al., 1994]. Post-proliferatively, in vitro and in vivo the osteoblasts express increasing levels of alkaline phosphatase enzyme activity requisite for maturation of the ECM in preparation for mineral deposition. Subsequently, increased expression of the non-collagenous ECM proteins, osteocalcin and osteopontin, characterizes onset of the mineralization period, the hallmark of expression of the mature bone cell phenotype. As a consequence, the osteoblast becomes embedded in a mineralized ECM and is designated as an osteocyte, the most mature among the osteoblast lineage. In vivo these cells reside in lacunae connected to other osteocytes through long cellular processes in canaliculi. In vitro, the osteocytes are among those cells associated with the mineralized nodules (Fig. 1) [Pockwinse et al., 1992].

Here we show cells expressing the osteoblast phenotype undergo programmed cell death during the mineralization stage. Cell death by apoptosis in mature bone cells associated with a mineralized ECM was directly demonstrated by in situ end-labeling and by degradation of DNA into oligonucleosomal fragments after gel electrophoresis. We also demonstrate, for the first time, selective expression (mRNAs) of genes associated with regulation of apoptosis during the growth and differentiation stages of primary diploid osteoblasts in vitro. These findings provide direct evidence for implicating apoptosis in osteogenesis that is relevant to regulation of bone formation in the adult skeleton.

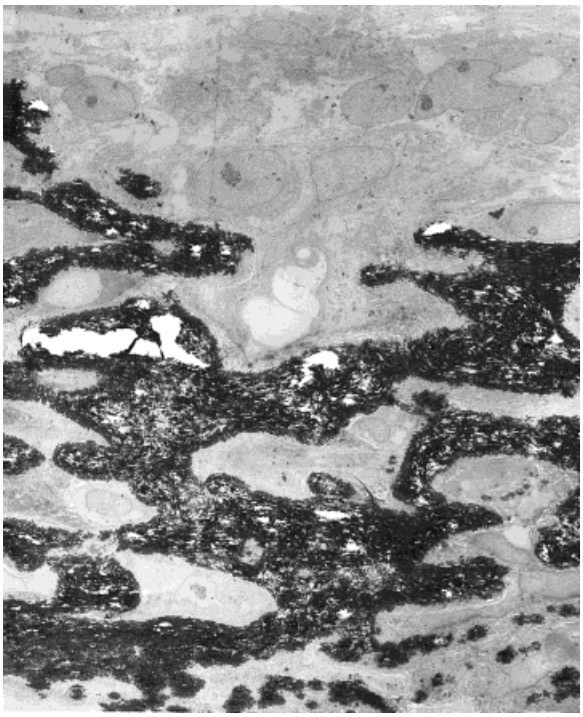

Fig. 1. In vitro formation of bone-like matrix by day-22 fetal rat calvarial derived osteoblasts. Cells were cultured as described in Materials and Methods. Electron micrograph of a cross-section of the bone nodule showing numerous osteoblasts on the surface that underlie transitional osteocytes surrounded by mineralized matrix. Prepared as described in Pockwinse et al., 1992. Magnification ×2,500; courtesy of S.M. Pockwinse.

MATERIALS AND METHODS

Cell Culture

Calvaria were excised from 21-day fetal rats and the dura and periosteum removed. Cells were obtained from the calvaria by three sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN)/0.25% trypsin (Gibco, Grand Island, NY). The cells of the third sequential digestion were maintained in culture. Cells were plated at a density of 6.5×10^5 as primary cultures or 3.6×10^5 cells/dish (100 mm or six-well) (Corning, Corning, NY), passaged at confluency and grown in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS). From day 5 onward, the medium was supplemented with 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate to support matrix deposition and mineralization. In some experiments, cells were passaged at confluency and replated at the densities noted above. Depending on the experiment, 10^{-7} M dexamethasone (Dex)

(Sigma Chemical Co., St. Louis, MO) was added to the medium of passaged cells for the first three feedings to induce and accelerate nodule formation; however, those cells harvested during the growth period for gene expression studies did not contain Dex. Medium was replenished every 2 days.

Characterization of Osteoblast Proliferation and Differentiation

For each experiment, differentiation was monitored by histochemical staining for alkaline phosphatase (APase) activity, mineralization and matrix production, and by RIA for osteocalcin production. APase was detected after incubating the cells for 30 min at room temperature with shaking in 10 mM Tris HCl, pH 8.4, containing 20 mg/ml disodium napthol AS-MX phosphate and 40 mg/ml fast red TR salt (Sigma Chemical Co). Mineralization was assayed by von Kossa staining of cultures (30 min in 3% $AgNO_3$) [Clark, 1981]. Cells and matrix were revealed by staining with .05% Toluidine blue. Secreted osteocalcin was quantitated by radioimmunoassay procedure using reagents described previously in detail [Gundberg et al., 1984]. Osteocalcin measurements reflect the extent of osteoblast differentiation by levels of synthesized osteocalcin that correlate to mineralization of the ECM [Aronow et al., 1990]. To determine proliferation, cells were grown on 22-mm Thermanox coverslips (USA Scientific, Waltham, MA) and incubated with triated thymidine (^{3}H-thymidine) (New England Nuclear, Boston, MA) for 24 h at a concentration of 1 µCi/ml. Cells were then rinsed twice in ice-cold PBS and fixed in absolute methanol (−20°C). Coverslips were then air dried overnight. Autoradiography was performed using Ilford K-5 emulsion as described by Baserga and Malamud [1969]. Exposures were for 5–7 days at 4°C.

RNA Isolation

Total cellular RNA was isolated from frozen cell pellets stored at −70°C by the Chirgwin procedure [Chirgwin et al., 1979]. RNA was resuspended in diethyl pyrocarbonate-treated water, quantitated by absorbance at 260 nm, and stored at −70°C. RNA samples were monitored with respect to representation of ribosomal RNA (18S and 28S) as internal standards, and the intactness of the RNA was ascertained by electrophoretic fractionation on 6.6% formaldehyde, 1% agarose gels, and ethidium bromide staining. For Northern analysis, 10 µg of total cellular RNA was fractionated as described above, transferred to Zetaprobe membranes (Bio-Rad, Rockville Centre, NY) and cross-linked to the membranes by UV irradiation for 1 min. Probes were labelled with (α-^{32}P)dCTP to a specific activity of at least 1×10^9 dpm/µg DNA using Prime-it II random primer labeling kit (Strategene, La Jolla, CA). Gene probes were gel purified inserts isolated from plasmid DNA in 1% low melting point agarose (Ausubel, 1989). Homologous inserts include p53 [Soussi et al., 1988], alkaline phosphatase [Zernik et al., 1990], collagenase [Quinn et al., 1990], and osteocalcin [Lian et al., 1989]. Non-homologous inserts include probes to murine c-fos [Miller et al., 1984], Bcl-2 [Nuñez et al., 1990], Bax (Oltvai et al., 1993], Bcl-X_L [Boise et al., 1993], Msx-2 [Catron et al., 1993], and to human histone H4 [Pauli et al., 1989], IRF-1 [Maruyama et al., 1989], and IRF-2 [Itoh et al., 1989]. The prehybridization and hybridization conditions were as described previously [Shalhoub et al., 1991]. Blots were exposed to preflashed XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) using a Cronex Lightning Plus screen at −70°C for differing times depending on probe. Autoradiograms were quantitated by scanning laser densitometry using a UVP Gel Documentation System (San Gabriel, CA). Data were expressed in arbitrary densitometric units after mRNA levels were normalized to the amount of 18S ribosomal RNA determined from the ethidium bromide-stained gel.

Isolation of DNA From the Cultured Cells

Cells were lysed in 10 mM Tris, pH 7.4, 1.5 mM EDTA, 1% SDS, and incubated with 200 µg/ml proteinase K overnight at 37°C [Kyprianou and Isaacs, 1988]. The DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), then twice with chloroform:isoamyl alcohol (24:1) and precipitated overnight at −20°C in 0.3 M final concentration NaAcetate and 2.5 volumes absolute ethanol. The samples were centrifuged 30 min at 4°C at 12,000*g* and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). The concentration of DNA was determined by absorbance at 260 nm.

In Situ End-Labeling (ISEL) of Apoptotic Cells

Cell cultures were fixed on the days indicated in 4% buffered paraformaldehyde for 10 min,

and stored in 0.1 M cacodylate until staining by the ISEL procedure as described by Gavrieli et al. [1992]. Briefly, cultures were rinsed 4 times, 2 min each with distilled H_2O (dH_2O), endogenous peroxidase blocked by incubation in 2% H_2O_2 for 5 min and rinsed again with dH_2O. The cultures were then incubated in terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim) buffer (TdT buffer) (30 mM Tris, pH 7.2, 140 mM NaCacodylate, 1 mM cobalt chloride), 0.3 enzyme units/µl TdT and biotinylated dATP (Gibco) at 37°C for 1 h. The reaction was stopped by removal of the TdT buffer and addition of termination buffer (TB = 2 × SSC, 300 mM NaCl, 30 mM NaCitrate) for 15 min at room temperature. The cells were then washed with dH_2O, incubated with 2% BSA for 10 min, and incubated with Avidin peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min at 37°C. Negative controls received the same procedure without the addition of TdT. Labeled cells were identified by incubation with diaminobenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 30 min until the reaction product developed.

Nucleosomal Fragmentation Assay

Ten-microgram aliquots of DNA isolated as described above were suspended in gel loading buffer containing 10 µg/ml ethidium bromide and electrophoresed on 1.8% agarose gels in 1× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid) at 5V per cm, then photographed under UV transillumination. Alternatively, DNA was end-labeled with [^{32}P]-dCTP as described by Rösl [1992]. Briefly, 1–10 µg of DNA was incubated with 0.5 µCi dCTP and 5U Klenow polymerase in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$ for 10 min at room temperature. The reaction was terminated by the addition of 10 mM EDTA. Unincorporated nucleotides were removed by centrifugation over quick spin columns or by ethanol precipitation. Total DNA recovered was determined by absorbance at 260 nm and total counts incorporated determined by scintillation counting. DNA was electrophoresed as described above, the gel dried for 3 h and exposed to Kodak XAR film for autoradiography.

RESULTS

Apoptosis Is Functionally Linked to Differentiation of the Osteoblast Phenotype

Individual apoptotic cells were localized in primary cultures of calvarial rat osteoblasts by a sensitive in situ end-labeling technique [Gavrieli et al., 1992]. During the proliferative period (near monolayer confluency) in these cultures, a low percentage of individual apoptotic cells is observed randomly throughout the cell layer as evidenced by ISEL staining (Fig. 2A and B). As nodules form, more apoptotic cells appear in the pre-mineralized multilayered cell nodule (Fig. 2C and D). With progressive development of the nodules and mineralization of the extracellular matrix (ECM), a marked increase in the proportion of cells in the nodule is labeled. The apoptotic cells are predominantly associated with the central areas of the nodule where mineral deposition is initiated (Fig. 2E and F). In late stage cultures after day 30, an increase in the number of apoptotic cells appears dispersed throughout the entire mineralized nodule (Fig. 2G and H) and some are still detected in the internodular regions. Higher magnification (Fig. 2I) clearly shows strong intranuclear staining reflecting chromatin fragmentation.

This in situ labeling method can detect necrotic cells, since it relies on the availability of free 3′-OH termini of DNA; and in necrosis, the DNA is degraded at random intervals [Wijsman et al., 1993]. Thus, we verified that the cell death we observe during osteoblast differentiation is due to apoptosis and not necrosis by demonstrating that DNA is degraded into oligonucleosomal-sized fragments characteristic of apoptosis. These fragments could not be visualized after agarose gel electrophoresis and ethidium bromide staining, except in the mature fully mineralized cultures after day 28 (data not shown). Because the number of apoptotic cells was relatively low compared to the total number of viable cells throughout the cell layer, especially during the early and mid stages of osteoblast maturation, we used the more sensitive method of end-labeling the isolated DNA with [^{32}P]dCTP to detect nucleosomal degradation.

Figure 3A shows the autoradiographic detection of nucleosomal fragmentation of DNA isolated from cells harvested at different stages of osteoblast differentiation. Osteocalcin synthesis, a marker of the mature osteoblast phenotype, is presented as an indicator of the extent of osteoblast differentiation in this experiment (Fig. 3B). On day 7, the DNA ladder was clearly evident reflecting the in situ labeled cells throughout the cell layer at confluency shown

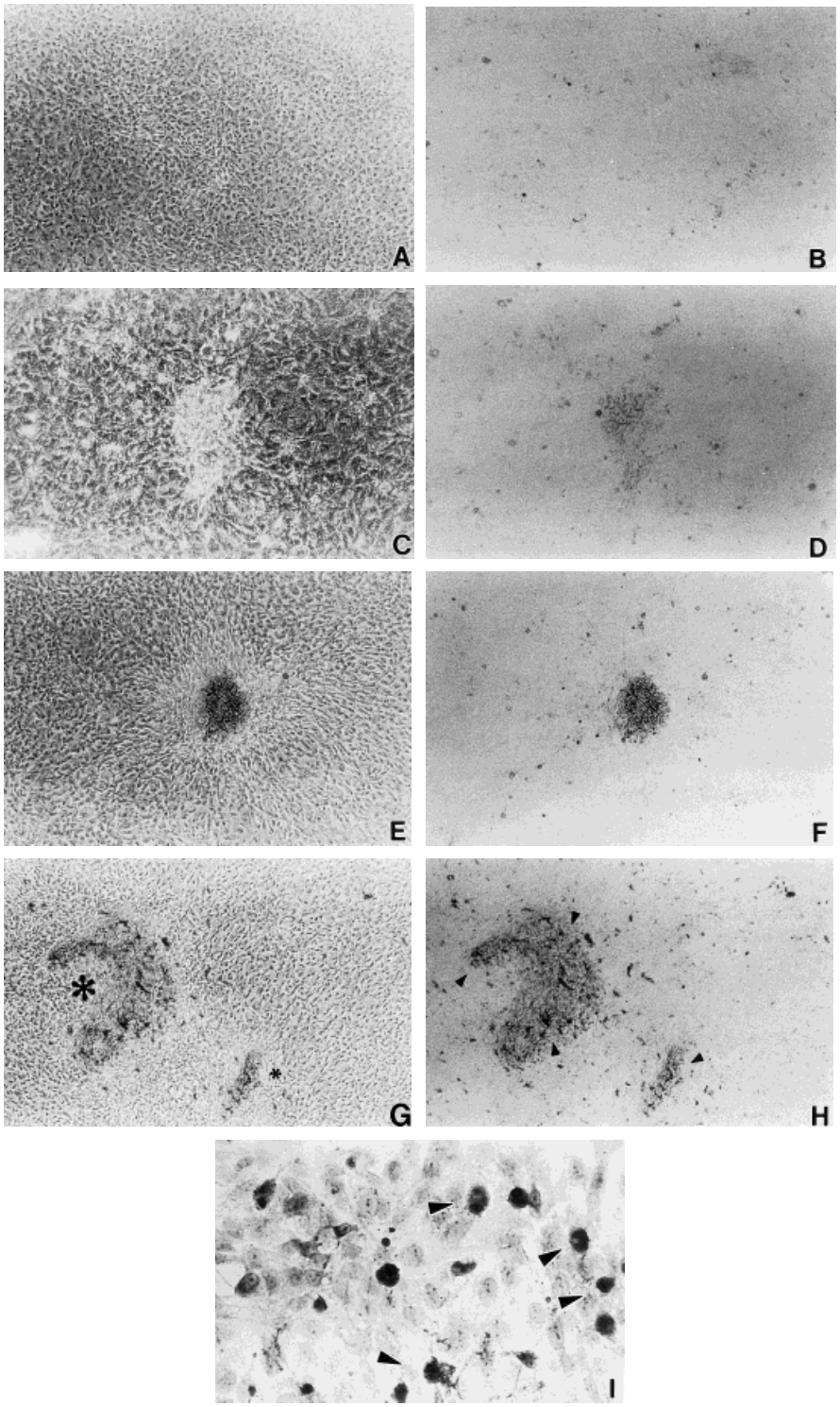

Fig. 2. Localization of apoptotic cells in cultures of fetal rat calvaria during the development of bone tissue-like organization. Fetal rat osteoblast were grown in culture for varying lengths of time, then fixed and labelled with TdT to identify apoptotic cells as described in Materials and Methods. All of the cultures depicted here were obtained from the same experiment in which the cells were at monolayer confluency on day 10 (A, B), began multilayering day 14 (C, D), and had initiated nodule mineralization by day 22 (E, F), which was well developed by day 29 (G, H). **A:** Day 10, ×6.3 magnification, phase contrast showing a confluent monolayer of cells. **B:** Bright field micrograph of the area shown in A revealing scattered apoptotic labelled cells (represented by black nuclei). **C:** Day 14, ×10 phase contrast of developing nodule (center). **D:** Bright field micrograph (×10) of the area shown in C showing numerous labelled cells in the region of cellular multilayering. **E:** Day 22, ×6.3 phase contrast and (**F**) bright field micrograph showing a small mineralizing nodule with numerous labelled cells associated distinctly with the nodular area. **G:** Day 29, ×6.3 phase contrast of a large, well-developed nodule (large asterisk) and a smaller one (small asterisk). **H:** Bright field micrograph of the area represented in G showing apoptotic cells predominantly associated with the nodule (surrounded by arrowheads). **I:** Higher magnification (×32) of labelled cells to demonstrate intranuclear staining of the dying cells. In several cells, the chromatin is condensed (arrowheads).

in Figure 2A,B. This representation of nucleosomal fragments remained constant in the pre and early mineralized stages of nodule formation (day 14–25 cultures). By day 29, with ongoing extracellular matrix mineralization, a dramatic increase in nucleosomal fragments was observed. The DNA isolated from cultures on day 29 appears partially cleaved into nucleosomes; however, distinct oligonucleosomal bands are resolved with lighter exposure of the autoradiograph (not shown). In multiple experiments, this pattern of DNA degradation is evident at these later points. This reflects a significant increase in the number of cells initiating or undergoing apoptosis during the period of rapid accumulation of mineral throughout the nodule (see Fig. 2G and H). On day 34, there appears to be a marked increase in nucleosomal degradation (Fig. 3, lane 34). At this time point, all nodules throughout the culture are completely mineralized. An apoptotic cell population on day 34 is clearly evident in the floating (non-adherent) cells that appear in media (Fig. 3, lane 34f).

We addressed the relationship of apoptosis to osteoblast differentiation, which occurs concomitant with extracellular mineralization, as opposed to cell senescence during the 5-week culture period. Cells grown in the absence of β-glycerophosphate (βGP) exhibit delayed mineralization of the nodules (Fig. 4) and developmental expression of the osteoblast phenotype. This is reflected by the low levels of osteocalcin secreted into the media (Fig. 5). Very few apoptotic cells are observed in the non-mineralized nodule (Fig. 4B) when compared to a mineralized nodule of the same size and duration of culture (see Fig. 2E and F). In the non-mineralized cultures, the appearance of apoptotic cells in the internodular monolayer region of the culture is similar to that observed in mineralizing cultures. Figure 5 shows that nucleosomal degradation is non-detectable in the minus βGP-treated cultures on day 25 to 29 (non-mineralized cultures) when compared to mineralized cultures on these days (see Fig. 3). The nucleosomal ladder observed on days 14 through 29 reflects the apoptotic cells throughout the internodular regions of the culture and in association with the multilayered nodule formed by day 22.

A second experimental approach was pursued to confirm the relationship of osteoblast apoptosis to differentiation. In the absence of ascorbic acid, collagen synthesis is low, cells do not form nodules (Fig. 4C and D), alkaline phosphatase activity is minimal [Aronow et al., 1990] and, therefore, no mineral is deposited because of the lack of formation of an organized and competent ECM. The 100-fold reduction in osteocalcin secreted in media on day 29 from these cultures (Fig. 5) reflects that maturation of the osteoblast is inhibited. Examination of nucleosomal fragmentation in cells grown in the absence of ascorbic acid reveals that, although there is some nucleosomal degradation evident on days 7 to 14 (data not shown), this does not persist. No enhancement of DNA fragmentation is observed on day 29 (Fig. 5). Therefore, inhibiting the development of the mature osteoblast phenotype by inhibiting nodule formation and mineralization inhibits apoptosis of the cells. These findings suggest a linkage between apoptosis and development of mature bone-like tissue formation marked by nodule formation, and to a greater extent mineralization of the nodule ECM.

To establish further that apoptosis is related to differentiation of the cells to the mature osteoblast phenotype, we grew cells in conditions that enhance differentiation. Supplementation of the culture medium with Dex for the first three medium changes, days 3, 5, and 7 after plating, results in accelerated nodule formation and mineralization (Fig. 6). A 7-fold increase in secreted osteocalcin is observed in the Dex-treated cultures on day 20 compared to the -Dex reflecting the extensive formation of mineralized nodules. Analysis of nucleosomal ladder formation shown in Figure 6 reveals that the extent of apoptosis in the day-12 cultures (beginning of nodule formation) is equivalent in the presence or absence of Dex. However, an increased nucleosomal fragmentation occurs with mineralization by days 16 and 20 when osteoblast differentiation is accelerated by Dex. In the experiment represented here, the development and mineralization of nodules was more rapid in the Dex-treated group than in the primary cultures presented in Figure 3. In both experiments, the onset of apoptosis detected by the in situ labeling coincides with the onset of mineralization. Thus, the accelerated maturation of the nodules in this experiment is paralleled by an increasing incidence of apoptosis through time in these cultures. These observations support the concept that apopto-

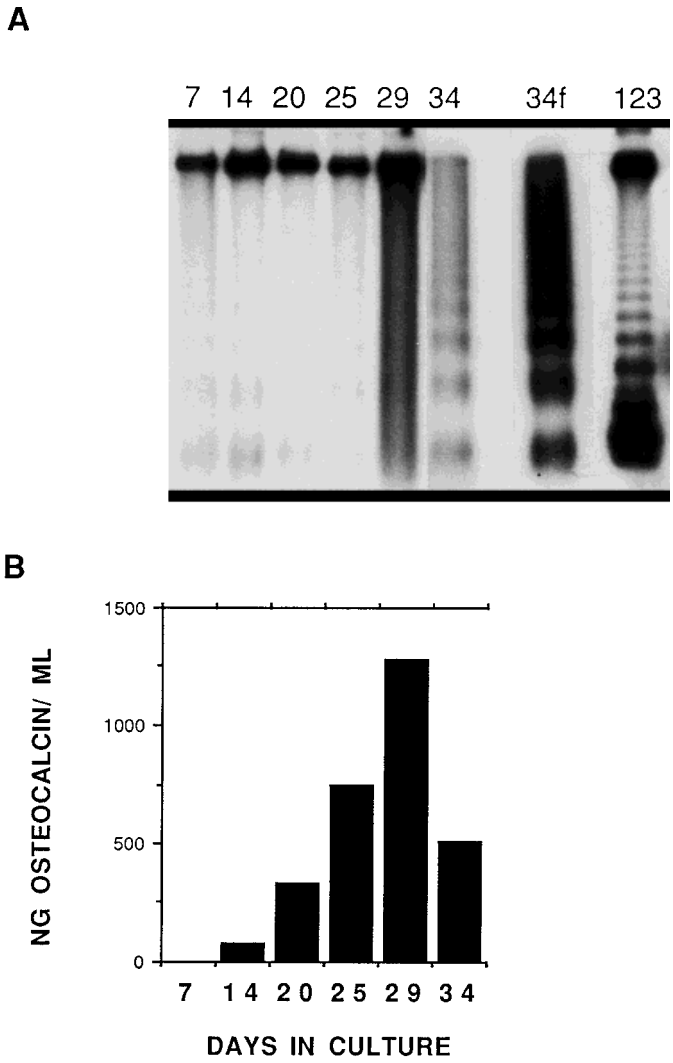

Fig. 3. Nucleosomal degradation in cultured fetal rat calvarial cells during development of the osteoblast phenotype. **A:** Autoradiography of end-labeled DNA after gel electrophoresis of equal amounts of DNA (200 ng) reveals low-level endonucleosomal degradation during growth (day 7), early nodule formation (day 14), and the matrix maturation period (days 20–25), up to the stage of advanced mineralization of the ECM (day 29). A significant level of the cell layer DNA on day 34 is represented in oligonucleosomal bands. **Lane 34f** represents DNA isolated from cells found floating in the culture medium on day 34. Since the same amount of DNA was loaded in each lane, lane 34f appears overloaded only because the majority of the DNA from the detached cells is fragmented. With a shorter exposure, distinct ladder bands are observed; however, the longer exposure is necessary to reveal the endonucleosome degradation in the cells attached to the dish (days 7 through 25). **Lane 123** represents DNA size markers. **B:** Osteocalcin synthesis (μg/ml secreted in media, 48-h accumulation) was monitored to reflect the osteoblast differentiation. Note a 56% decrease in osteocalcin on day 34 in the apoptotic stage from day 29.

sis is essential for bone-like tissue organization.

Parameters of Gene Expression Associated With Osteoblast Apoptosis

We have observed the selective appearance of apoptotic cells during osteoblast differentiation initially dispersed throughout the cell layer in proliferating cultures. Later, in differentiated cell cultures, apoptotic cells are concentrated to mineralized areas. On this basis we examined the expression of genes that regulate programmed cell death as a function of development of the osteoblast phenotype. Analysis of gene expression characterizing the growth period (Fig. 7A) and subsequent stages of differentiation (Fig. 7B) reveal the following distinctions. The period of active proliferation is

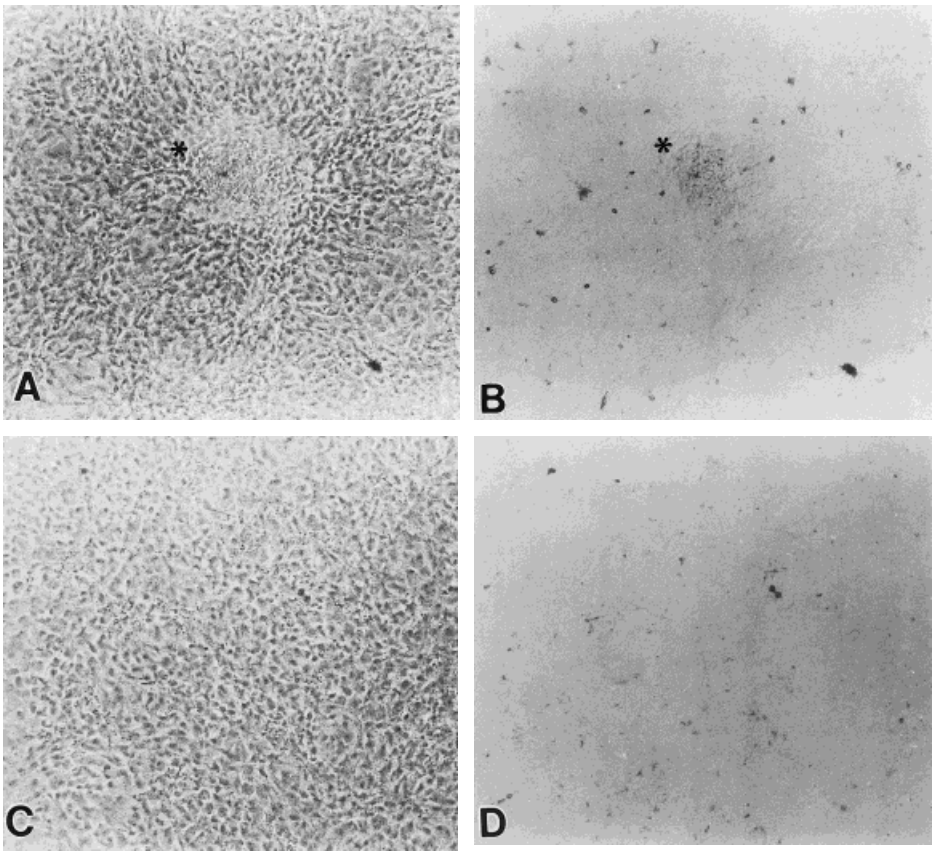

Fig. 4. Detection of apoptotic cells (TUNEL procedure) in cultures of fetal rat calvarial osteoblasts, which did not differentiate. **A:** Phase contrast and (**B**) bright field micrographs of day-28 osteoblasts maintained in MEM with 10% fetal calf serum and ascorbate, but lacking supplementation with 10 mM β-glycerol phosphate. Asterisk indicates representative nodule throughout the cultures that are not mineralized. **C:** Phase contrast and (**D**) bright field of day-18 osteoblasts maintained in the absence of ascorbic acid with media containing only 10% FCS. Note that nodules have not formed in these cultures. ×6.3.

reflected by expression of the histone H4 gene (coupled to DNA synthesis) and cdc2 and cdk2, two cell cycle regulatory proteins, on days 1 and 8. After monolayer confluency (day 8), levels of these mRNAs decline. Notably, a slight increase in histone gene expression occurs on day 14, reflecting the completion of cellular multilayering to form the nodules and again on day 27. Higher levels of c-fos, IRF-1, and IRF-2 (Fig. 7A) are observed on days 1 and 8 compared to the post-proliferative differentiating stage (days 14–22). We observe the expression of c-fos and IRF-1 as well as IRF-2 to be significantly higher on day 27 than in the proliferating period (days 1 and 8). The increases in mRNA levels from day 22 to 27 are 5.3 (c-fos), 6.0 (IRF-1), and 4.1 (IRF-2) fold (see Fig. 8, which presents quantitation of the Northern blot analyses). In contrast, cdc2 mRNA levels were undetectable and only a small increment in histone and cdk2 mRNA levels (1.2-fold) are observed. These findings suggest that the rise in mRNAs of c-fos, IRF-1, and IRF-2 may be more related to apoptotic events than to cell growth. This suggestion is substantiated by analysis of cell proliferation throughout the culture period (Fig. 9).

Autoradiography after incorporation of ^{3}H-thymidine by the cells at different stages of differentiation shows uniform labeling of cells throughout the plate during the growth period (Fig. 9a, day 2) and labelled cells were associated with expansion of the nodule in the matrix maturation phase (Fig. 9b, day 14). In contrast, only a small population of proliferating cells were observed in the heavily mineralized cultures and these cells were localized to the apex of the mineralized nodule with a few peripheral labelled cells (Fig. 9c). Electron microscopic

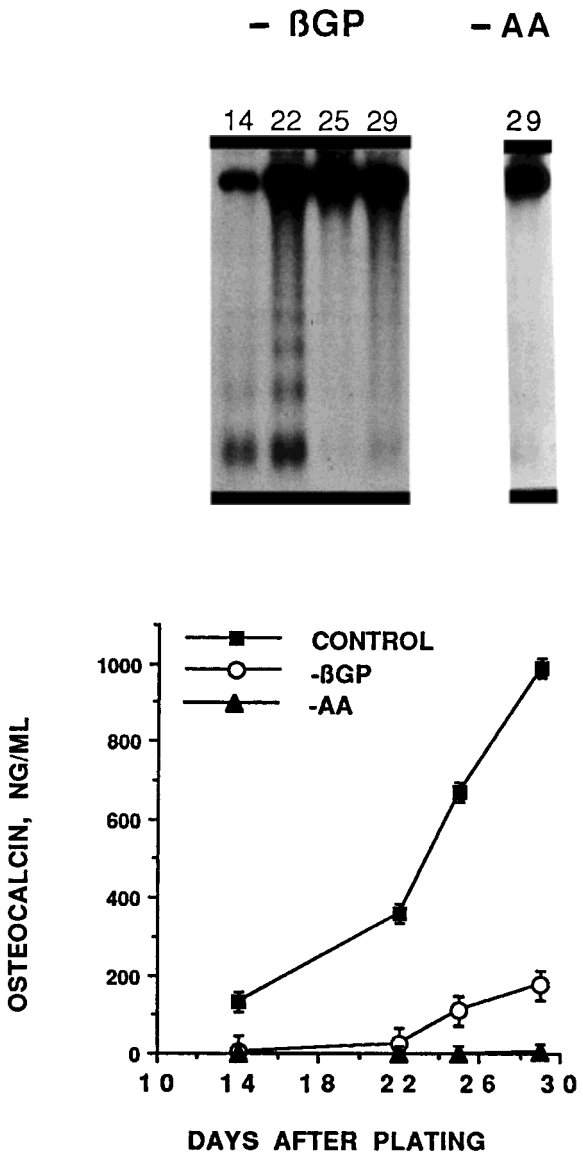

Fig. 5. Nucleosomal fragmentation of DNA isolated from cells cultured in conditions that inhibit development of the osteoblast phenotype. Rat calvaria-derived osteoblasts are grown in the absence of β-glycerophosphate (-βGP), which promotes mineral deposition or absence of ascorbic acid (-AA), a requirement for matrix synthesis and expression of the osteoblast phenotype. **Top:** DNA isolated from cells grown in these conditions shows that DNA degradation is evident on days 14 to day 22, but little thereafter. Induced levels on day 29 were not observed as shown in control cultures presented in Figure 2. The end-labeling of the samples in Figure 2 and in these samples was done at the same time, as well as exposure for autoradiography for the same length of time. Hence, the degree of apoptosis indicated in Figure 2 and here may be directly compared from the intensity of the bands on the autoradiogram. **Bottom:** Secreted osteocalcin measured in 48-h conditioned media by a radioimmunoassay reflects the extent of osteoblast differentiation.

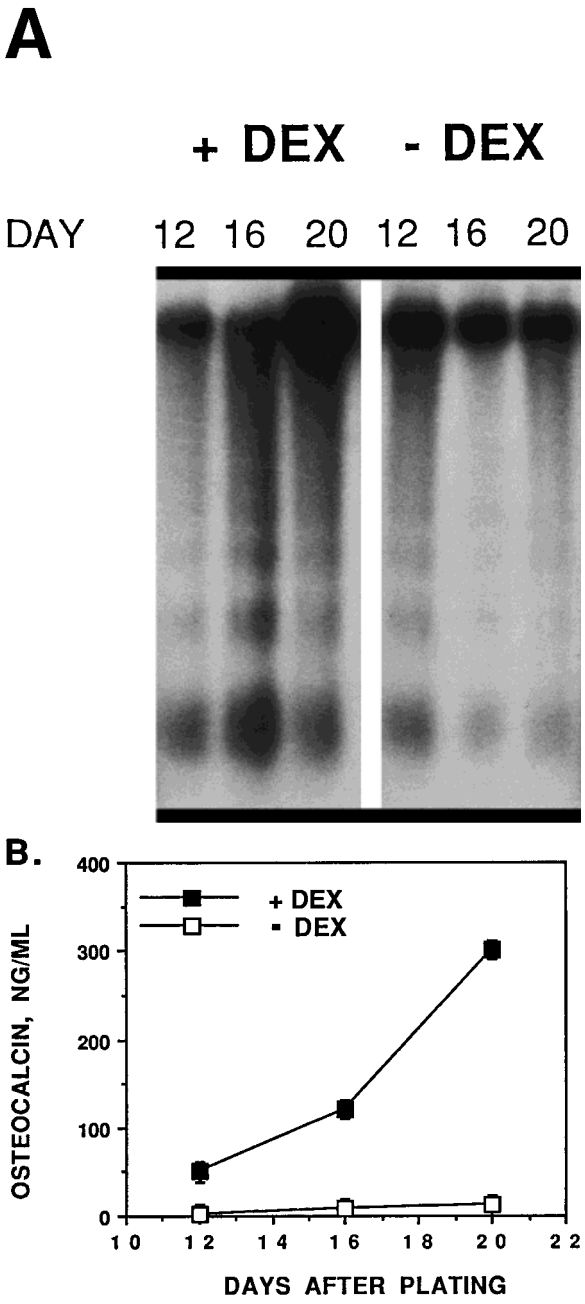

Fig. 6. Apoptosis is increased in osteoblasts cultured in conditions that support phenotype differentiation. Cells were subcultivated on day 5 of primary culture and grown in the presence of dexamethasone from days 3 through 9 (required for osteoblast differentiation in passaged cells) or in the absence of dexamethasone and harvested at the indicated days. **A:** Autoradiography after electrophoresis of equal amounts of DNA (250 ng) reveals increasing amounts of nucleosomal breakdown through time in culture (+ Dex). **B:** Osteocalcin synthesis (secreted in media, 48 h) reflects osteoblast differentiation and mineralization of nodules formed in + Dex treated cultures of passaged cells compared to control (− Dex) cultures.

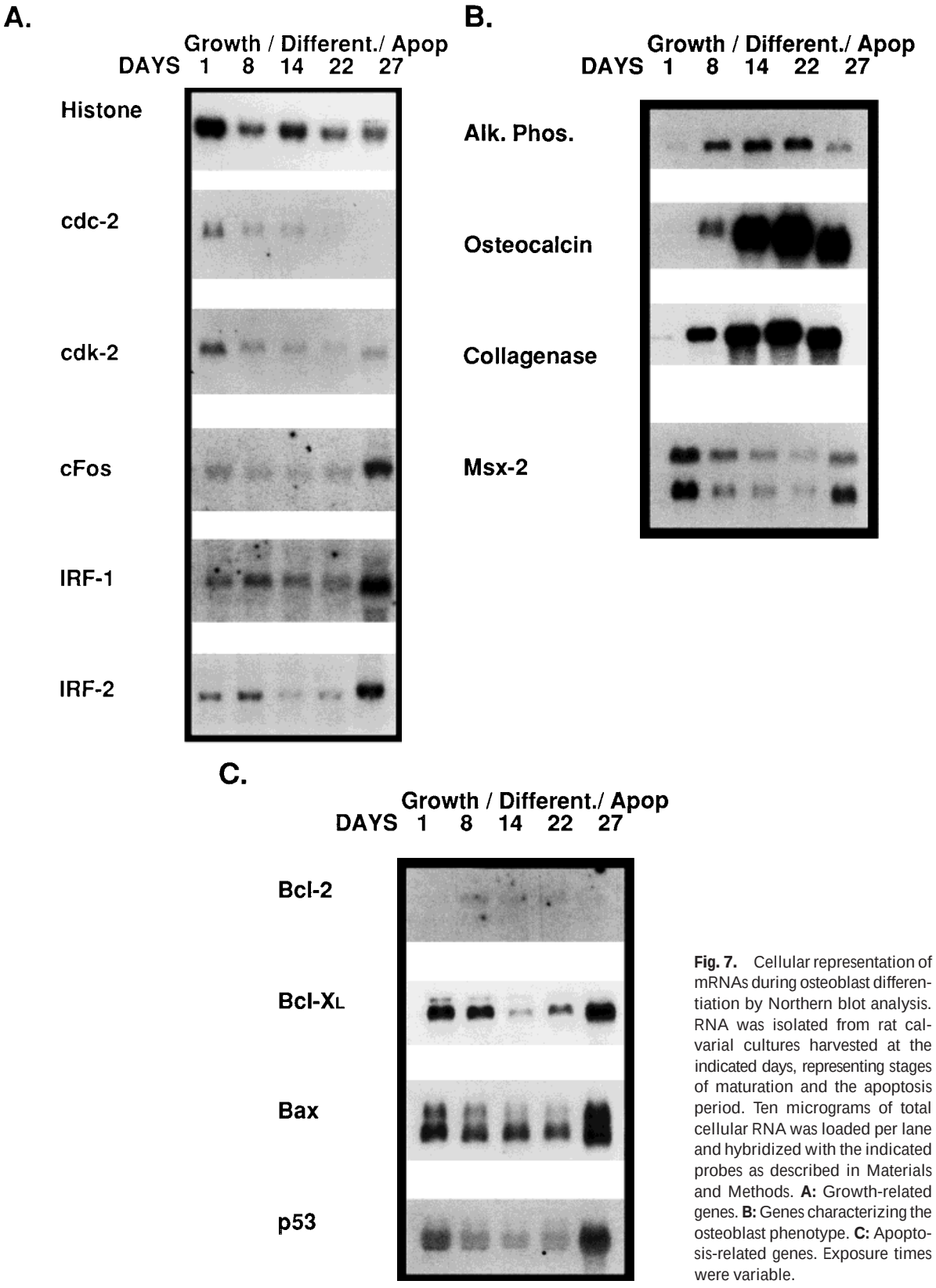

Fig. 7. Cellular representation of mRNAs during osteoblast differentiation by Northern blot analysis. RNA was isolated from rat calvarial cultures harvested at the indicated days, representing stages of maturation and the apoptosis period. Ten micrograms of total cellular RNA was loaded per lane and hybridized with the indicated probes as described in Materials and Methods. **A:** Growth-related genes. **B:** Genes characterizing the osteoblast phenotype. **C:** Apoptosis-related genes. Exposure times were variable.

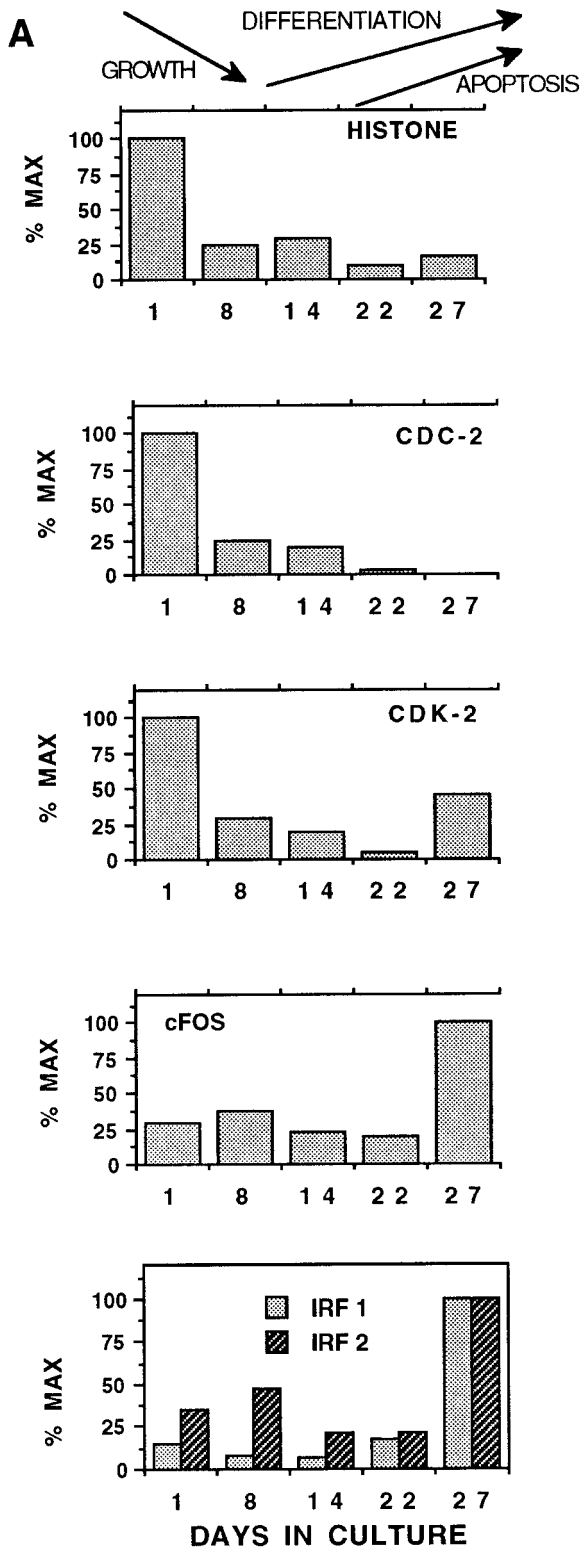

Figure 8.

cross-sections of the mature mineralized nodule were examined to reveal dividing and apoptotic cells in relation to tissue organization.

Expression of the bone phenotypic genes (Fig. 7B) shows increasing alkaline phosphatase expression from day 8 to 22, reflecting the matrix maturation period. The onset or initiation of mineral deposition on day 14 is reflected by the 10-fold rise in osteocalcin from day 8. Collagenase expression also increases post-proliferatively and is maintained at a significant level throughout osteoblast differentiation. In isolated osteoblasts, Msx-2 mRNA, represented by two transcripts 2.2 and 1.4 kd [Bell et al., 1993], is downregulated after proliferation [Hoffmann et al., 1994], but in these studies we show Msx-2 becomes significantly increased on day 27. We find that the 1.4 Kd transcript of Msx-2, which declined sharply after day 1, is increased 6-fold from day 22 to day 27 while the 2.2 Kd band increases 3.3-fold. Taken together, the relatively few ^{3}H-thymidine labelled cells on day 25 (Figure 9) and the modest increase in histone H4 expression on day 27 (from day 22) suggest that the significant increased expression on day 27, of Msx-2, c-fos, and the IRFs, may be associated primarily with the apoptotic cell population.

Expression of genes specifically associated with regulation of programmed cell death were examined throughout the course of osteoblast differentiation (Fig. 7C). The Bcl-2 gene, an inhibitor of apoptosis, could be detected at low levels by Northern blot analysis. The normalized densitometric values plotted in Figure 8 show the highest Bcl-2 mRNA levels were observed on day 8 through 22, with lower mRNA levels on days 14 and 27; but Bcl-2 was nondetectable on day 1, the peak of proliferation. Bcl-X_L, another gene related to suppression of apoptosis and associated with long-term postmitotic cells [Boise et al., 1993], was well represented in the osteoblast preparations. We observed high Bcl-X_L mRNA levels during the

Fig. 8. Quantitation of Northern analyses of osteoblast expressed genes. Profiles of expression for each gene are represented as percent maximal expression. Each bar in a gene profile is the average of 2 determinations (one of these is shown in Fig. 7), each normalized to 18S ribosomal RNA from the ethidium bromide-stained gel. Autoradiograms were quantified by scanning densitometry using a UVP Gel Documentation System. **A:** Growth-related genes. **B:** Genes characterizing the osteoblast phenotype. **C:** Apoptosis-related genes.

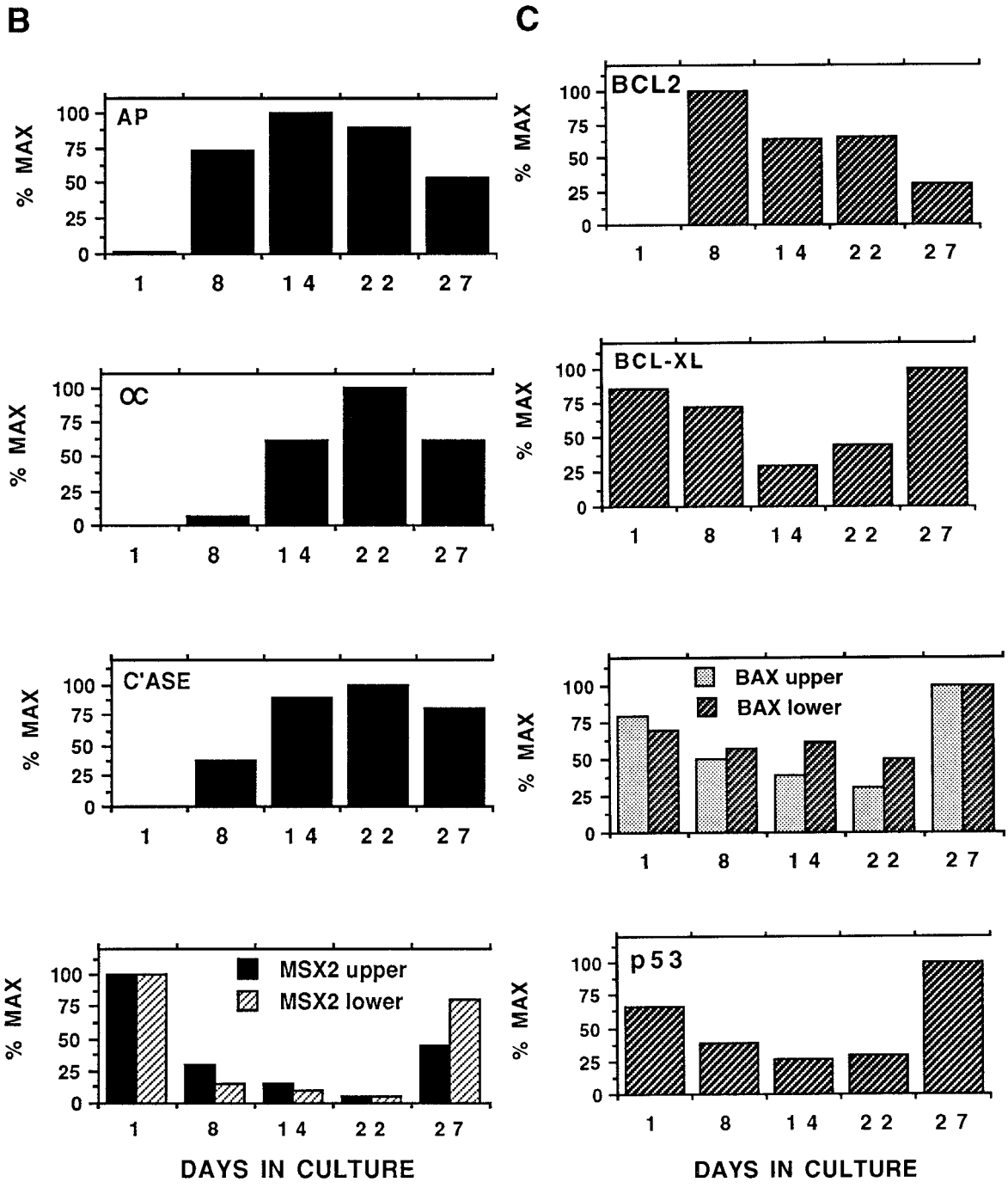

Fig. 8. Continued.

growth period, days 1 and 8, a decline and then an increase on day 27 to maximal expression (from 42% day 22 to 100% day 27). Bax, which is well documented to promote apoptosis or accelerate programmed cell death, was observed to be expressed at maximal levels in the day-27 cultures. Day 1 mRNA levels of both transcripts were 70–79% of the day-27 expression with lower levels at intervening times (Fig. 8C). Expression of the tumor suppressor, p53, also an inducer of apoptosis, parallels mRNA levels of Bax with the highest levels observed on day 1 (66%) and day 27 (100%).

DISCUSSION

Our results suggest that cell death by apoptosis is a component of the developmental expression of the osteoblast phenotype. A small per-

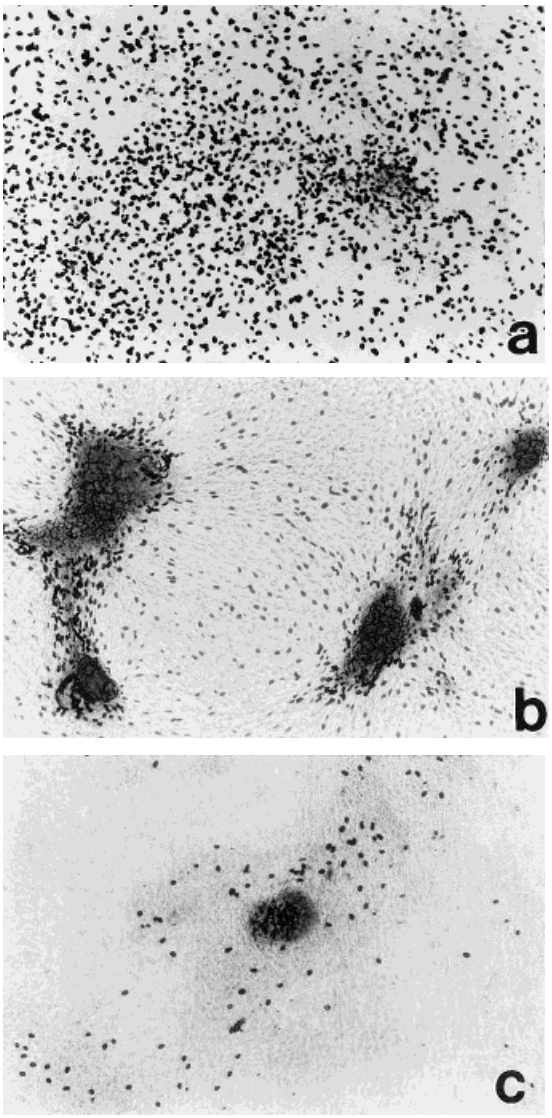

Fig. 9. Autoradiography of ^{3}H-thymidine pulsed rat osteoblast cultures. Proliferating cell populations are distributed on: (**a**) Day 7, uniformly throughout the cultures; (**b**) day 14, primarily in the area of the growing nodules; (**c**) day 25, sparsely at the periphery of the nodule and a few cells clustered at the apex of the mineralized nodule. Cells were labelled with ^{3}H thymidine and fixed as described in Materials and Methods. Cell layers were stained with Toluidine blue to reveal nodules and cells. B was stained for alkaline phosphatase activity (grey area) to better show the nodule boundary.

centage of dying cells are observed throughout the culture in the early stage of growth. These cells may represent a subpopulation that became apoptotic when isolated from the bone tissue or following proliferation in vitro, lacked additional signals necessary for survival. These signals may derive from a surrounding ECM, intercellular communication, and cytokines or growth factors that associate with the mineralized bone ECM [Birnbaum and Wiren, 1994; Tang et al., 1996; Breen et al., 1994; Hauschka et al., 1986], providing a unique microenvironment for osteoblast viability. In other systems, apoptosis is induced by growth factor withdrawal or hormone ablation [Batistatou and Greene, 1993; Kyprianou and Isaacs, 1988; Duke and Cohen, 1986]. Accumulation of an ECM might function to support survival of at least a subpopulation of cells in these cultures. This requirement of an ECM for cell viability is not unique to osteoblasts. The studies of Ishizaki et al. [1995] show that cells cultured at low density in the absence of serum and exogenous signalling molecules undergo PCD, suggesting that they need extracellular signals to avoid apoptosis. This is particularly relevant to survival of mammary epithelial cells [Boudreau et al., 1995]. Thus apoptosis may be activated in some osteoblasts isolated from the tissue stripped of their ECM environment.

Apoptosis becomes a prominent feature of the in vitro culture in the late mineralization stage during the development of the mature osteoblast phenotype, the differentiated osteocyte. We confirmed biochemically that the cell death observed in these cultures is indeed due to apoptosis and not necrosis by observing the degradation of DNA into oligonucleosomal fragments. The relationship of apoptosis to the final stage of osteoblast differentiation, when the cells mature into osteocytes surrounded by mineralized matrix (Fig. 1), is supported by our findings that nucleosomal fragmentation is not evident under conditions that do not support bone-like tissue formation (Figs. 4, 5, and 6). The absence of ascorbic acid from the media to prevent nodule formation and the absence of β-glycerophosphate during the culture period to inhibit ECM mineralization inhibit apoptosis. Conversely, acceleration of differentiation by dexamethasone leads to an earlier increase in the number of apoptotic cells.

The significant increase in the representation of apoptotic cells distributed largely throughout the nodule is coincident with extensive mineralization of the extracellular matrix. The sustained increase in collagenase in differentiated cells reflects both viability of the cultures and the contribution of collagenase to extracellular matrix "editing." Our findings provide the first in vitro evidence for programmed cell death occurring in osteoblast/osteocyte-like

cells associated with the mineralizing matrix. This may be a consequence of the inability of all the osteoblasts synthesizing matrix in a nodule in vitro to further differentiate because the cell cannot reside in a lacuna surrounded by mineralized matrix and interact through cell processes with other osteocytes. Our results in vitro are consistent with in vivo reports of morphological changes in osteocytes that suggest apoptosis in bone [Zimmerman, 1992; Jande and Bélanger, 1973; Frost, 1960; Bronckers et al., 1996a]. In vivo the majority of dying osteocytes are phagocytized by osteoclasts [Bronckers et al., 1996a], which are lacking in our cultures. Quite commonly in vitro apoptotic cells are released into the media if they cannot all be phagocytized by neighboring cells. This is apparent in our model where the floating cells have DNA degraded into nucleosomal fragments.

The upregulation of several markers of apoptotic events during the mineralization period may reflect activation of signalling cascades implicated in programmed cell death. Early cell cycle and apoptotic events are related [reviewed in Meikrantz and Schlegel, 1995]. Expression of c-fos appears to precede terminal differentiation and cell death [Smeyne et al., 1993; Preston et al., 1996]. C-fos also precedes differentiation of osteoblasts [McCabe et al., 1996; Machwate et al., 1995]. Both in vivo "gain of function" studies in transgenic mice [Grigoriadis et al., 1993] and in vitro expression and antisense studies in vitro [McCabe et al., 1996], demonstrate c-fos is a key regulator of osteoblast maturation. The involvement of cdc2 and cdk2 kinase complexes have been postulated among the many biochemical mediators activated during cell death. These enzymes are key components of the cell cycle machinery with cdc2 regulating the G2/M transition. Cdc2 mRNA is precipitously downregulated post-proliferatively and is not detected on day 27 consistent with the low representation of dividing cells. In contrast, cdk2, which acts during G1 and S phase in mammalian cells [Nurse, 1994; Hunter and Pines, 1994], increased on day 27, which may reflect a role in apoptotic events. The significant upregulation of c-fos, IRF-1 and 2, and p53, appear to be related to apoptotic events during the mineralization period since other genes involved in proliferation are maintained at lower levels on day 27 (histone, cdc2). The slight increase in histone gene expression and localization of the ^{3}H thymidine labeled cells to the nodule suggest that some compensatory proliferation is ongoing, but not enough to account for the dramatic upregulation of the genes we observe. p53 blocks cells in the G_1 phase of the cell cycle and has been associated with the propensity of some cells to undergo apoptosis [Clarke et al., 1993; Lowe et al., 1993; Kastan, 1993]. p53 expression is normally induced by DNA damage and it appears essential for the attended block in cell cycle progression and the consequential apoptosis [Lane, 1992; Donehower and Bradley, 1993]. Both p53 and IRF-1 may converge to regulate cell cycle control [Tanaka et al., 1996], but each may operate distinct apoptotic pathways [Tamura et al., 1995]. The disproportionate increases in IRF-1 and IRF-2 may be related to p53 independent pathways associated with deregulation of growth control [Tamura et al., 1995; Tanaka et al., 1994].

The homeodomain protein Msx-2, which is significantly upregulated on day 27, may be involved in a signaling mechanism leading to apoptosis during osteoblast differentiation. Msx-2 expression in vivo requires epithelial-mesenchymal interactions and is related to limb, mandible, and tooth development [MacKenzie et al., 1992; Davidson et al., 1991]. The region of mesenchymal cells expressing the Msx-2 gene overlaps with areas of developmentally programmed cell death [Davidson et al., 1991; Coelho et al., 1991; Graham et al., 1993], but precedes apoptosis in a precisely co-localized pattern within rhombomeres 3 and 5 that become depleted of neural crest cells [Coelho et al., 1991; Graham et al., 1993]. During in vitro growth of osteoblasts, Msx-2 is downregulated post-proliferatively. Thus, the increased levels of Msx-2 expression in the mineralization period may be related to apoptosis associated with cellular organization of the bone forming tissue. Notably, Msx-2 is a negative regulator of the bone specific osteocalcin gene [Towler et al., 1994; Hoffmann et al., 1994, 1996] consistent with osteocalcin downregulation during apoptosis.

The different profiles of expression for specific regulators of programmed cell death parallel the representation of apoptotic cells during development of the osteoblast phenotype. Bcl-2, which inhibits apoptosis [Alnemri et al., 1992; Wagner et al., 1993; Korsmeyer, 1992b], is detectable between days 8 and 22 when low numbers of apoptotic cells are evident throughout

the matrix maturation stage of osteoblast differentiation. Bcl-2 may function through a mechanism that has been attributed to providing protection against oxidative damage [Hockenbery et al., 1993]. The osteoblasts in the mineralizing cultures may be experiencing modifications in oxidative metabolism analogous to hypertrophic chondrocytes in relation to cartilage matrix calcification [Matsumoto et al., 1988; Shapiro et al., 1982] that leads to apoptosis of the hypertrophic chondrocytes [Hatori et al., 1995].

Bcl-X_L (an isoform of Bcl-X) is a Bcl-2-related gene product that heterodimerizes with the death-promoting Bax [Oltvai et al., 1993] and can inhibit apoptosis as effectively as Bcl-2 in thymocytes [Chao et al., 1995], but cell-type specific effects have been observed [Shimizu et al., 1995; Gottschalk et al., 1994]. We show Bcl-X_L exhibits a reciprocal profile of expression compared to Bcl-2 during osteoblast differentiation. The highest expressed levels of Bcl-X_L are observed when cells are first isolated from the tissue during their growth period prior to the accumulation of an ECM. Bcl-X_L functions by forming ion-channels, a finding that may be of particular relevance to osteocytes that maintain bone integrity by communication with each in a mineralized environment. Dissociation from the bone matrix may trigger Bcl-X_L to sustain cell viability [Minn et al., 1997]. By day 14, an ECM has accumulated accounting for 10–15% of the total cell layer protein [Aronow et al., 1990]. Thus, Bcl-X_L may play a more active role during development of the osteoblast phenotype to protect cells from cell death until they can support themselves within an extracellular matrix. Bcl-X_L expression decreases until day 27, then is induced to high levels when extensive apoptosis in mineralizing nodules is ongoing. Perhaps this increase reflects protection of a subset of cells in an apoptotic environment. These questions will be resolved in future studies identifying expression of Bcl-2 and Bcl-X_L at the single cell level.

Expression of Bax mRNA parallels Bcl-X_L levels with high levels detected when the cells are first isolated from the tissue and again in the apoptotic period. Overexpressed Bax counters the death repressor activity of Bcl-2 [Oltvai et al., 1993], which is not detected during the initial growth period. Thus signalling cascades leading to activation of Bax may occur when non-dividing cells are removed from the in vivo environment without sufficient support for survival during the growth period, and again when cells need to be deleted as a consequence of the final stage of maturation to the terminally differentiated osteocyte in the day-27 cultures.

Apoptosis is a fundamental process of embryonic bone development, e.g., deleting domains of mesenchymal cells to formation of the digits and long bone growth and deleting the hypertrophic zone for long bone growth in vivo. Our demonstration of apoptosis during differentiation of cultured osteoblasts provides a model for addressing molecular mechanisms and signalling pathways that may regulate decisions for osteoblast-osteocyte differentiation as well as osteocyte survival. We interpret our findings, demonstrating the relationship between programmed cell death and osteoblast differentiation, to support the hypothesis that apoptosis of cells in the osteoblast lineage is an important component of bone tissue development. Furthermore, recognition of apoptosis as a fundamental component of maintaining normal bone integrity suggests that derangements in the ability of osteoblasts to undergo apoptosis might be implicated in skeletal pathologies and aging.

ACKNOWLEDGMENTS

The authors thank Drs. P. Vaughn and D. Hushka for valuable discussions, J. Rask for manuscript preparation, and J. Landrie for photographic assistance. This publication was made possible by N.I.H. grants AR39588 and AR42262. The contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. Maureen P. Lynch and Casey Capparelli contributed equally to this work.

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