

High Osteoblastic Activity In C3H/HeJ Mice Compared to C57BL/6J Mice Is Associated with Low Apoptosis in C3H/HeJ Osteoblasts

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Abstract. This study sought to confirm that osteoblasts of C3H/HeJ (C3H) mice, which have higher differentiation status and bone-forming ability compared to C57BL/6J (B6) osteoblasts, also have a lower apoptosis level and to test whether the higher differentiation status and bone-forming ability of C3H osteoblasts were related to the lower apoptosis. C3H mice had 50% fewer ($P < 0.01$) apoptotic osteoblasts on the endocortical bone surface than B6 mice as determined by the TUNEL assay. Primary C3H osteoblasts in cultures also showed a 50% ($P < 0.05$) lower apoptosis level than B6 osteoblasts assayed by acridine orange/ethidium bromide staining of apoptotic osteoblasts. The lower apoptosis in C3H osteoblasts was accompanied by 22% ($P < 0.05$) and 56% ($P < 0.001$) reduction in the activity of total caspases and caspases 3/7, respectively. C3H osteoblasts also displayed greater alkaline phosphatase (ALP) activity ($P < 0.001$) and higher expression of *Chfa1*, type-1 collagen, osteopontin, and osteocalcin genes ($P < 0.05$ for each). To assess if an association existed between population apoptosis and the differentiation status (ALP-specific activity) and/or bone-forming activity (insoluble collagen synthesis), C3H and B6 osteoblasts were treated with several apoptosis enhancers (tumor necrosis factor- α , dexamethasone, lipopolysaccharide, etoposide) and inhibitors (parathyroid hormone, insulin-like growth factor I, transforming growth factor β 1, estradiol). Both ALP ($r = -0.61$, $P < 0.001$) and insoluble collagen synthesis ($r = -0.61$, $P < 0.001$) were inversely correlated with apoptosis, suggesting that differentiation (maturation) and/or bone-forming activity of these mouse osteoblasts were inversely associated with apoptosis. In conclusion, these studies support the premise that higher bone density and bone formation rate in C3H mice could be due in part to lower apoptosis in C3H osteoblasts.

Key words: Apoptosis — Differentiation — Osteoblast — Genetic regulation — Mice

Osteoporosis, which afflicts more than 20 million people yearly in the United States alone, is due in part

to inadequate bone formation to compensate for the increase in bone resorption that is frequently associated with aging and/or estrogen deficiency. Bone formation rate is controlled not only by the number of active osteoblasts but also by the activity of each individual osteoblast. Understanding of the mechanism(s) determining the osteoblastic activity, and consequently the bone formation rate, would not only yield important insights into the pathogenesis of osteoporosis but also provide a rational basis for designing effective osteogenic therapeutics for osteoporosis and other bone-wasting diseases.

During the course of our investigation into the genetic factors controlling peak bone mass, we found that C3H/HeJ (C3H) inbred mice had a 53% higher peak bone density in the femur than C57BL/6J (B6) inbred mice [1]. Our subsequent investigations revealed that the higher bone density in C3H mice was due largely to a higher bone formation rate, which was caused exclusively by an increase in osteoblast activity rather than increased osteoblast proliferation and/or recruitment [2–4]. Thus, this pair of inbred strains of mice would be useful in our investigation into the regulatory mechanisms of osteoblastic activity. Our recent studies have suggested that the more mature osteoblasts in C3H mice not only showed greater bone-forming activity but also appeared to have a lower apoptosis level than those in B6 mice [4]. It was envisioned that low apoptosis in osteoblasts could lead to a longer overall life span, which might result in a cell population with more mature and functionally active osteoblasts. Consequently, we postulate that osteoblasts in C3H mice have a lower apoptosis level than those in B6 mice and that the lower apoptosis in C3H osteoblasts may in part contribute to the greater differentiation status and bone-forming activity of C3H osteoblasts compared to B6 osteoblasts.

The objectives of this study were threefold. First, we sought to determine that osteoblasts of C3H mice indeed exhibited lower apoptosis than osteoblasts of B6 mice in thin bone sections *in vivo* since isolated osteo-

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blasts do not always mimic *in vivo* intrinsic differences. Second, we sought to confirm that the differentiation status and/or bone-forming activity of C3H osteoblasts were indeed greater than those of B6 osteoblasts by assessing the expression of several marker genes of mature osteoblasts with real-time polymerase chain reaction (PCR). Third, we sought to determine if the higher differentiation status (i.e., alkaline phosphatase [ALP]-specific activity) and bone-forming activity (i.e., insoluble collagen synthesis) of C3H osteoblasts compared to B6 osteoblasts were associated with their lower apoptosis.

Materials and Methods

Animals

C3H inbred mice were purchased from Jackson Laboratories (Bar Harbor, ME), and B6 mice were obtained from a colony maintained in Dr. Wes Beamer's laboratory (Bar Harbor, ME). The mice were maintained on a 4% fat-containing diet (Harlan Teklad, Madison, WI) and tap water *ad libitum* and housed in a controlled environment with a 12 hour/12 hour light/dark cycle within the animal facility at the Jerry L. Pettis Memorial V.A. Medical Center (Loma Linda, CA). Pregnant mice were given a 6% fat-containing diet (Harlan Teklad) until the end of lactation. The experimental design and the animal component of the research protocol were reviewed and approved by the animal care and use committee of the Jerry L. Pettis Memorial V.A. Medical Center prior to the initiation of the studies.

Cell Cultures

Primary osteoblasts were isolated from the calvariae of neonatal C3H and B6 inbred mice ($n = 16-20$). Briefly, hair, skin, and soft tissues were first trimmed off and the brain and other nonbony tissues removed from the calvariae. The parietal bones of calvariae were cut into small pieces and digested with 0.1% trypsin for 10 minutes at 37°C. After removal of the supernatant, the remaining bone chips were rinsed with phosphate-buffered saline (PBS) and treated with 2 mg/mL of crude collagenase (Sigma, St. Louis, MO) at 37°C for 90 minutes on a shaker to release the bone cells. Isolated cells were grown in 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium with 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 2.5 µg/mL of amphotericin B (Mediatech, Herndon, VA) at 37°C in a humid atmosphere of 5% CO₂. After approaching confluence, cells were trypsinized and stored in a liquid nitrogen tank until use. Cells of passages 2-4 were used in these studies.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated Diuridine Triphosphate Nick End Labeling (TUNEL) Assay

In vivo apoptosis of bone cells was assessed by TUNEL assay (Promega, Madison, WI) on paraffin-embedded femoral sections of 8-week-old mice. Femoral thin sections were first incubated with proteinase K (20 µg/mL) for 20 minutes. The DNA fragments of apoptotic cells were end-labeled by biotinylated nucleotide mix through the action of the TdT. The labeled cells were identified by a linker (avidin-horseradish peroxidase) and a chromogen/substrate (3,3'-diaminobenzidine/H₂O₂) of the peroxidase. The stained sections were counterstained with hematoxylin and coverslipped with per-

mount. Negative controls without the TdT were included in each assay. The percentage of apoptotic cells was calculated by dividing the number of labeled apoptotic cells by the number of counted cells. A total of at least 400 osteoblasts were counted in each section, and five to seven sections per group were analyzed.

Acridine Orange/Ethidium Bromide (AO/EtBr) Staining

The relative percentage of apoptotic cells in each culture was assessed by AO/EtBr dual staining. Briefly, cell layers were washed once with PBS and stained with the nucleic acid-binding dye mix containing 100 µg/mL AO (Sigma) and 100 µg/mL EtBr (Sigma) for 20 minutes. The cells were immediately examined by fluorescence microscopy [5]. Apoptotic cells had bright, condensed nuclei, which were also irregular in shape. For each sample, at least 400 cells/well and three wells/sample were counted. The percentage of apoptotic cells was determined by dividing the number of apoptotic cells by the number of counted cells.

Caspase Activity Assay

To determine the activity of caspases, a total of 5×10^3 osteoblasts/well of a 96-well plate were cultured in a 0.1% FBS condition for 24 hours. Two different commercial caspase activity assay kits were used to determine caspase activity. For the activity of total caspases (i.e., caspases 2, 3, 6-10), we used the Roche Diagnostics (Palo Alto, CA) fluorescent caspase assay kit with a fluorescent microplate reader. To determine the activity of caspases 3/7, the commercial Caspase-Glo™3/7 Assay kit (Promega) was used, with a microplate luminometer (EG&G Berthold, Bundoora, VIC, Australia).

Cell death enzyme-linked immunosorbent assay (ELISA)

For correlation studies, the apoptosis level was assessed with a commercial colorimetric Cell Death ELISA kit (Roche Diagnostics), which measured the amount of histone-associated DNA fragments as an index of cell death (apoptosis). Primary mouse osteoblast cultures (2×10^4 /cm²) were treated with either apoptosis inducers (i.e., tumor necrosis factor [TNF- α , 10⁻⁹ M], dexamethasone [DEX, 10⁻⁷ M], lipopolysaccharide [LPS, 25 µg/mL], and etoposide [10⁻⁴ M]) or inhibitors (i.e., insulin-like growth factor I [IGF-I, 50 ng/mL], parathyroid hormone [PTH, 5 \times 10⁻⁸ M], estradiol [E₂, 10⁻⁹ M], and transforming growth factor- β 1 [TGF- β 1, 100 pg/mL]) in either 0.1% or 1% FBS with or without ascorbate (50 µg/mL) for 1 or 3 days. Absorbance (a measure of cell death) was measured at 550 nm with a spectrophotometer.

ALP activity assay

To measure ALP activity, mouse osteoblasts (2×10^4 /cm²) were grown in 0.1% or 1% FBS for 1-3 days. Cell layers were immediately extracted with 0.1% Triton X-100. ALP activity was measured by calculating the change of absorbance at 405 nm over the assay time period with paranitrophenylphosphate as the substrate [6]. ALP activity was normalized by protein level using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Insoluble Collagen Synthesis Assay

We measured the production of insoluble collagen to determine bone matrix-forming activity of osteoblasts by a procedure described previously [7]. Briefly, mouse osteoblasts (2×10^4 /cm²) were grown for 3 days in a 1% FBS condition in the presence of ascorbate (50 µg/mL, Sigma). After incubation, cell layers were

Table 1. Sequence of individual PCR primers used for real-time PCR analysis

Gene	Forward primer	Reverse primer
<i>Cbfa-1</i>	5'-CAGACCAGCAGCACTCCATA-3'	5'-CAGCGTCAACACCATCATTTC-3'
<i>Colla1</i>	5'-GCCTCCCAGAACATCACCTA-3'	5'-AGTTCCGGTGTGACTCGTG-3'
<i>Opn</i>	5'-CTGTTGCCAGCTTCTGAGCA-3'	5'-TGTGGCTCTGATGTTCCAGGCT-3'
<i>Bglap1</i>	5'-CTCTCTCTGCTCACTCTGCT-3'	5'-TTTGTAGGCGGTCTTCAAGC-3'
<i>Bsp</i>	5'-AAGCAGCACCGTTGAGTATGGG-3'	5'-ACCCTCGTAGCCTTCATAGCCA-3'
<i>β-Actin</i>	5'-CAGGCATTGCTGACAGGATG-3'	5'-TGCTGATCCACATCTGCTGG-3'

fixed with picric acid: 37% formaldehyde (3:1) containing 4.8% acetic acid for 1 hour. After fixation, cell layers were air-dried and insoluble collagen was stained with 0.1% Sirius red F3BA (Aldrich, Milwaukee, WI) in picric acid for 1 hour. After washing with 0.01N HCl, the bound dye was released from the cells with 0.1N NaOH. The intensity of released dye was read at 550 nm with a spectrophotometer.

Real-Time PCR

Mouse osteoblasts ($1 \times 10^4/\text{cm}^2$) were plated in six-well plates and grown for 3 days in the presence of 1% FBS and ascorbate (50 $\mu\text{g}/\text{mL}$). At the end of each experiment, total RNAs were immediately extracted with the commercial RNeasy Mini kit (Qiagen, Valencia, CA), and the contaminating genomic DNA in the RNA samples was digested with DNase (Invitrogen, Carlsbad, CA). DNase-treated RNAs were reverse-transcribed into cDNA and kept at -20°C until use. Real-time PCR assays were performed with a commercial QuantiTectTMSYBR[®] green PCR kit (Qiagen) and the ABI PRISM 7000 detection system (Applied Biosystems, Foster City, CA). PCR conditions consisted of an initial 10-minute hot start at 95°C , followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 1 minute, and a final step of melting curve analysis from 60°C to 95°C . The sequence of the PCR primer sets of each gene of interest is listed in Table 1. The specificity of each primer set was first tested by checking the size of PCR products on 1% agarose gels before use. Data normalization was performed against an endogenous housekeeping gene control (*β-Actin*), and the normalized values were used to calculate the relative fold change between the two groups by the threshold cycle method.

Statistical Analysis

The data were presented as mean \pm standard deviation (SD). Student's *t*- or paired *t*-test was used to analyze any difference between the two mouse strains with the Microsoft (Redmond, WA) Excel for Windows 2003 version on a personal computer. For regression analyses, we used Statistica statistical software (Statsoft, Tulsa, OK). Differences were considered significant at $P < 0.05$.

Results

Comparison of Osteoblast Apoptosis between C3H and B6 Mice

To confirm that the C3H mice indeed had a lower apoptosis level in osteoblastic cells than the B6 mice, we first examined femoral bone samples ($n = 5-7/\text{group}$) from previous studies for apoptotic osteoblasts by the TUNEL staining assay. The number of osteoblasts per specimen was about 476 ± 140 . As summarized in

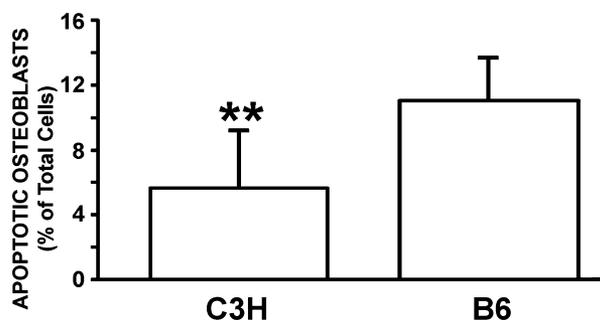


Fig. 1. An *in vivo* comparison of the percentage of apoptotic osteoblasts on C3H and B6 femoral sections (8 weeks old) by the TUNEL staining assay ($n = 5-7$). Data presented as mean \pm SD. ** $P < 0.01$ by Student's *t*-test.

Figure 1, the femoral bone sections of C3H mice had approximately 50% fewer apoptotic osteoblasts on the endocortical bone surface than those of B6 mice. These TUNEL data are consistent with our previous *in vitro* fluorescence-activated cell sorting (FACS) data [4], which showed 30–40% fewer apoptotic osteoblasts in C3H cultures than in B6 cultures isolated from mice at birth or 6 weeks of age.

To further confirm that C3H osteoblasts indeed had lower apoptosis than B6 osteoblasts, we measured the percentage of apoptotic osteoblasts isolated from the parietal bones of the calvariae from 1-day-old pups using the AO/EtBr staining assay to differentiate apoptotic from living cells. The AO/EtBr staining assay has been widely used to identify apoptotic cells [8–10]. Once cells become apoptotic, the nuclei become acidic and able to trap AO dye. Figure 2A shows that C3H osteoblasts (left panel) had a lower number of condensed fluorescent apoptotic nuclei than B6 osteoblasts (right panel). The number of apoptotic osteoblasts detected by AO/EtBr in the primary C3H osteoblast cultures was also approximately 50% lower ($P < 0.05$) than in primary B6 osteoblast cultures (Fig. 2B). These findings indicate that the *in vivo* difference in apoptosis in osteoblasts between C3H and B6 mice was maintained in isolated osteoblasts *in vitro* for at least four passages under our culturing conditions.

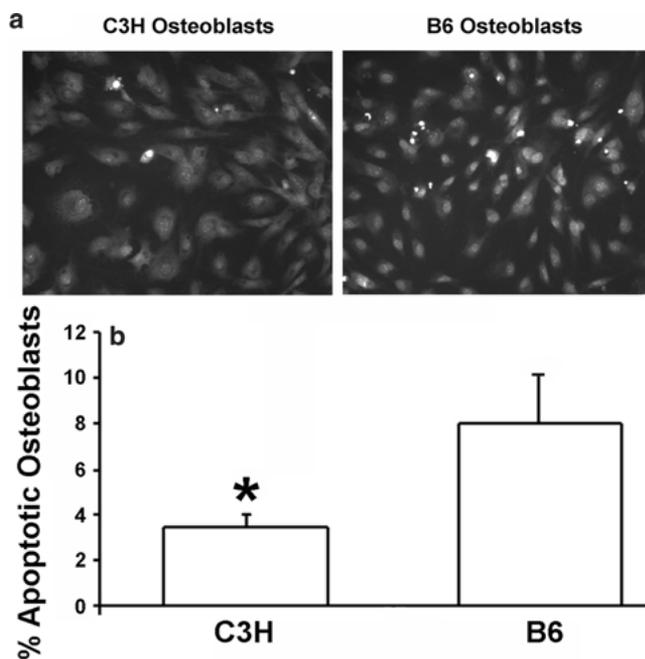


Fig. 2. An *in vitro* comparison of the percentage of apoptotic osteoblasts identified by AO/EtBr dual staining from C3H and B6 osteoblast cultures. C3H (left) and B6 (right) osteoblasts were isolated from newborn pups and cultured for 24 hours in the presence of ascorbate. Under a fluorescent microscope, apoptotic osteoblasts were identified by condensed fluorescent nuclei that were irregular in shape (A). The percentage of apoptotic cells was calculated by dividing the number of apoptotic cells by the number of counted cells (B). * $P < 0.05$ by Student's *t*-test.

Comparison of Basal Caspase Activity between C3H and B6 Osteoblasts

Apoptosis occurred as a consequence of triggering complex signal cascades, which usually involve activation of caspases [11–13]. Thus, we next determined whether there was a corresponding difference in caspase activity between primary C3H and B6 osteoblasts. Figure 3 shows that primary C3H osteoblasts showed 22% ($P < 0.05$) lower total caspase (i.e., caspases 2, 3, 6–10) activity and 56% ($P < 0.001$) lower caspase 3/7 activity than B6 osteoblasts. These findings further support the premise that C3H osteoblasts have significantly lower apoptosis than B6 osteoblasts and suggest that the difference in apoptosis between C3H and B6 osteoblasts is in part mediated through lower basal caspase activity, particularly caspases 3/7.

Comparison of the Differentiation Status of C3H and B6 Osteoblasts

Our previous studies indicated that osteoblasts in C3H mice had higher differentiation status and bone-forming activity than those in B6 mice [4]. Our hypothesis

predicts that lower apoptosis in C3H osteoblasts would lead to an overall higher differentiation status. Hence, we compared the differentiation status of primary C3H osteoblasts with that of primary B6 osteoblasts by measuring the basal expression levels of various osteoblastic differentiation markers in these osteoblasts. Since ALP-specific activity has been regarded as an osteoblastic differentiation marker, we first determined whether there was a significant difference in ALP activity (normalized against cellular protein) between the pooled neonatal osteoblasts of C3H and B6 mice. In agreement with our previous studies [4], the ALP-specific activity in C3H osteoblasts was indeed significantly greater than that in B6 osteoblasts by more than twofold (i.e., 11.1 ± 0.78 vs. 3.69 ± 0.46 mU/mg protein, $P < 0.001$, six replicates).

We next measured and compared the expression of several osteoblastic marker genes (i.e., *Cbfa-1*, type I collagen $\alpha 1$ [*colla1*], bone sialoprotein [*Bsp*], osteopontin [*Opn*], and osteocalcin [*Bglap1*]) by real-time PCR in primary osteoblasts of C3H and B6 mice. As shown in Figure 4, C3H osteoblasts expressed significantly higher levels of message transcripts for *Cbfa-1* (by 2.2-fold, $P < 0.01$), type 1 collagen $\alpha 1$ (by 1.6-fold, $P < 0.01$), osteopontin (by 3.1-fold, $P < 0.05$), and osteocalcin (by 2.3-fold, $P < 0.05$) than B6 osteoblast cultures. There was no significant difference in the expression of *Bsp* between the two mouse strains under our culturing condition. These findings are consistent with our previous *in vivo* observations that osteoblasts of C3H mice were generally more mature and exhibited greater bone-forming activity than osteoblasts of B6 mice [2–4] and support our hypothesis that lower apoptosis in osteoblasts could lead to higher overall differentiation status and/or greater bone-forming activity.

Association between Apoptosis and Differentiation Status and/or Bone-Forming Activity of Osteoblasts of C3H and B6 mice

To test our hypothesis that a reduction in apoptosis would lead to an increase in differentiation status in osteoblasts of C3H and B6 mice, we determined if there was a significant correlation between apoptosis and the differentiation status of primary C3H and B6 osteoblasts. We treated primary C3H and B6 osteoblasts with various apoptosis inducers (i.e., TNF- α , DEX, LPS, and etoposide) or inhibitors (i.e., IGF-I, TGF- $\beta 1$, E_2 , and PTH). As shown in Figure 5, there was a significant inverse correlation ($r = -0.61$, $P < 0.001$) between cell death and ALP-specific activity in these primary mouse osteoblasts. There was no significant difference in the slope of the ALP-specific activity vs. cell death plot between C3H and B6 osteoblasts, indicating that the relationship between the differentiation status (i.e.,

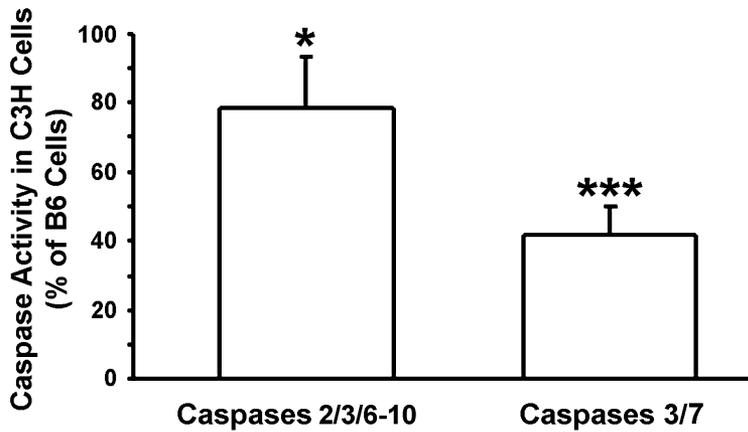


Fig. 3. A comparison of the activities of total caspases (2, 3, 6–10) and caspases 3/7 between C3H and B6 cell cultures. Pooled osteoblasts from newborn pups were cultured in 0.1% FBS for 24 hours. The values (mean \pm SD) for total caspase activity and for caspase 3/7 activity of B6 osteoblasts (six replicates per assay) were 39 ± 6 relative fluorescent units and $71,893 \pm 9,037$ relative light units per well, respectively. The experiment for caspase 3/7 activity was repeated twice and yielded very similar results. * $P < 0.05$, *** $P < 0.001$ by Student's *t*-test.

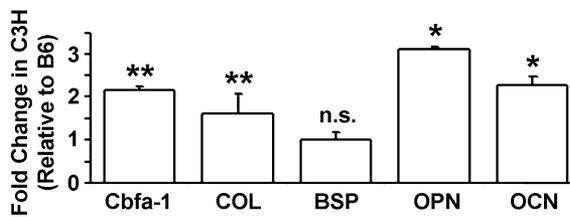


Fig. 4. A comparison of the expression of genes associated with differentiation/activity between C3H and B6 osteoblasts. Pooled neonatal osteoblasts were cultured for 3 days in the presence of ascorbate. Expression of the gene of interest was normalized by the mouse β -actin expression level, and normalized values were used to calculate the fold change. Data (mean \pm SD) are presented as the fold change in C3H relative to that in B6. The experiment was repeated, $n=4$ and yielded very similar results. * $P < 0.05$; ** $P < 0.01$; n.s., not significant by paired *t*-test.

ALP-specific activity) and apoptosis in C3H osteoblasts was not different from that in B6 osteoblasts.

We next sought to determine if there was also a similar association between apoptosis and the bone-forming activity of osteoblasts of these two inbred strains of mice. The production of insoluble collagen, an acceptable index of bone-forming activity of osteoblasts [14, 15], was measured. The basal apoptosis of these osteoblasts was again perturbed by treatment with the same apoptosis inducers (i.e., TNF- α , DEX, LPS, and etoposide) or inhibitors (i.e., IGF-I, TGF- β 1, E₂, and PTH) in the presence of ascorbate (to promote collagen synthesis) for 3 days. As shown in Figure 6, there was also a significant inverse correlation ($r = -0.61$, $P < 0.001$) between cell death and insoluble collagen production in these primary mouse osteoblast cultures. The slope of the insoluble collagen vs. cell death plot between C3H and B6 osteoblasts was also not significantly different, suggesting a similar relationship between apoptosis and bone-forming activity in osteoblasts of both C3H and B6 mice.

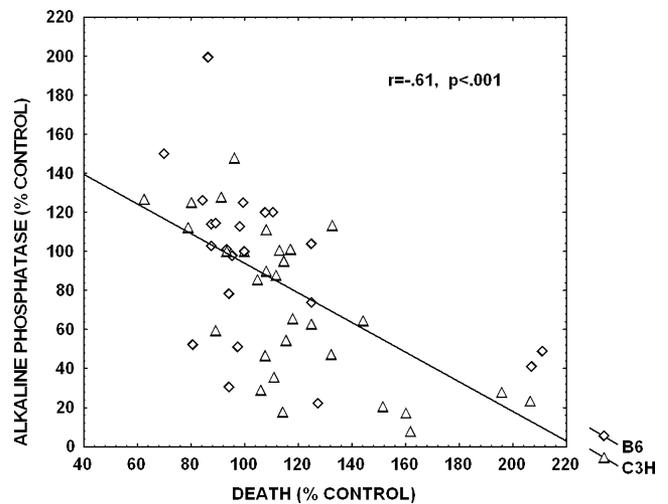


Fig. 5. A negative correlation between apoptosis and ALP-specific activity of C3H and B6 osteoblasts. Primary osteoblasts of 1-day-old C3H and B6 mice were treated with vehicle (basal), an apoptosis inducer (i.e., TNF- α , DEX, LPS, or etoposide), or an inhibitor (i.e., IGF-I, TGF- β 1, E₂, or PTH) to generate a range of population apoptosis. Apoptosis level was measured by a commercial cell death ELISA kit. Data are presented as percentage of untreated control. Because the slope of the plot for C3H osteoblasts was similar to that for B6 osteoblasts, data of both C3H and B6 osteoblasts were combined for correlation analysis. Diamonds, B6 osteoblasts; triangles, C3H osteoblasts.

Discussion

Our previous investigations have demonstrated that the higher peak bone density in C3H mice compared to B6 mice was attributed to a greater bone formation rate that was due largely to an intrinsically higher differentiation status and bone-forming ability in C3H osteoblasts [2–4]. During our search for a potential mechanism that might contribute to the higher differentiation status of C3H osteoblasts, we obtained FACS analysis data that C3H osteoblasts appeared also to

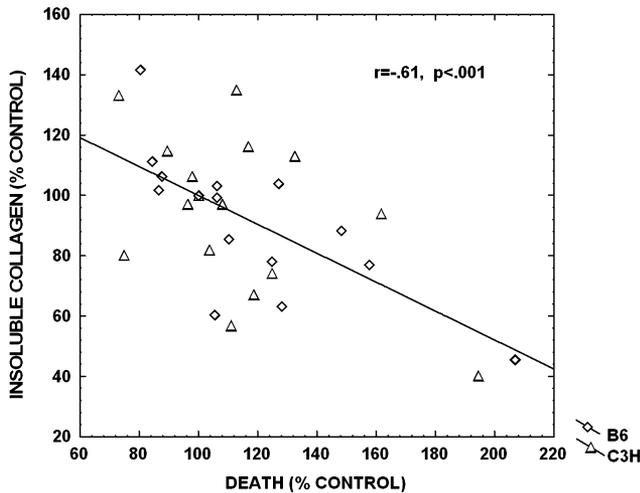


Fig. 6. A negative correlation between apoptosis and insoluble collagen production of C3H and B6 osteoblasts. Primary osteoblasts of 1-day-old C3H and B6 mice were treated with vehicle (basal), an apoptosis inducer (i.e., TNF- α , DEX, LPS, or etoposide), or an inhibitor (i.e., IGF-I, TGF- β 1, E₂, or PTH) to generate a range of population apoptosis. Apoptosis level was measured by a commercial cell death ELISA kit. Data are presented as percentage of untreated control. Because the slope of the plot for C3H osteoblasts was similar to that for B6 osteoblasts, data of both C3H and B6 osteoblasts were combined for correlation analysis. Diamonds, B6 osteoblasts; triangles, C3H osteoblasts.

have a significantly lower apoptosis level [4]. We envision that lower osteoblast apoptosis in C3H mice, which probably would result in an increase in the overall life span of their osteoblasts, could lead to an overall greater differentiation status and greater bone-forming activity compared with B6 osteoblasts. Accordingly, while the mechanisms responsible for the greater intrinsic differentiation status and bone-forming activity in C3H osteoblasts compared to B6 osteoblasts have not been elucidated, we postulate that the lower osteoblast apoptosis could at least in part contribute to the greater intrinsic bone-forming activity in osteoblasts of C3H mice.

In this study, we have obtained further *in vivo* and *in vitro* evidence confirming significantly lower apoptosis in C3H osteoblasts compared to B6 osteoblasts. Accordingly, we found that 8-week-old C3H mice had an approximately 50% lower percentage of apoptotic osteoblasts on the endocortical bone surface of femurs than B6 mice and that primary C3H osteoblasts of 1-day-old mice *in vitro* also had an approximately 50% lower percentage of apoptotic osteoblasts than primary B6 osteoblasts. Our previous FACS analysis also showed that primary osteoblasts from 6-week-old C3H mice had approximately 50% fewer apoptotic osteoblasts compared with B6 mice at the same age [4]. The fact that C3H osteoblasts also had approximately 50% lower basal caspase 3/7 activity compared to B6 osteo-

blasts is entirely consistent with the conclusion that the apoptosis in C3H osteoblasts was 50% lower than that in B6 osteoblasts. Thus, we conclude that the basal apoptosis in C3H osteoblasts is 50% lower than that in B6 osteoblasts. This 50% lower apoptotic rate should not be considered trivial since it represents a highly significant decrease (from 11% in B6 mice to 5.5% in C3 mice) in the percentage of apoptotic osteoblasts along the endocortical bone surfaces. Since osteoblasts of C3H mice at different test ages (i.e., 1-day-old, 6-week-old, and 8-week-old) exhibited 50% lower apoptosis than osteoblasts of B6 mice at each corresponding age, the difference in apoptosis between these two strains, like the difference in their bone-forming activity [4], is intrinsic to their osteoblasts, develops very early in life (probably during the embryonic period), and continues throughout the growth period.

With respect to the differentiation status and bone-forming activity of osteoblasts of these two mouse strains, we have previously reported that C3H osteoblasts had two- to threefold higher ALP-specific activity and showed an approximately fivefold greater bone nodule-forming ability than B6 osteoblasts [4]. Consistent with our previous data, this study showed that primary osteoblasts isolated from C3H mice expressed significantly higher levels of mRNA transcripts of several osteoblastic genes, i.e., *Cbfa-1*, type-I collagen α 1, osteopontin, and osteocalcin, than primary osteoblasts from B6 mice. This difference in differentiation status and bone-forming activity was not due to a difference in osteoblast number since our previous studies indicated that the population doubling time of isolated osteoblasts *in vitro* [4] and tetracycline-labeled bone surface length (i.e., bone-forming surface) *in vivo* [2, 3] were not different between the two mouse strains. Given the fact that the basal proliferation rate of C3H osteoblasts (by the [³H]-thymidine incorporation assay) was approximately twofold lower than that of B6 osteoblasts [4], the observed 50% lower apoptosis in C3H osteoblasts without a significant difference in population size indicated that the greater differentiation status and bone-formation activity of C3H osteoblasts could be attributed to a lower cell turnover (or an overall longer life span) of osteoblasts in C3H mice than B6 mice. Thus, although we did not measure the population life span of these two osteoblast populations in our studies, we favor the conclusion that the observed greater intrinsic bone-forming activity in the C3H osteoblast populations could at least in part be due to the overall increase in the percentage of the number of mature osteoblasts in the entire population as the consequence of an overall longer life span and lower apoptosis in C3H osteoblasts.

In strong support of our hypothesis that there is an association between the lower apoptosis and the greater differentiation status and bone-formation activity of

osteoblasts of these mouse osteoblasts, we found that there was a significant and inverse correlation between apoptosis and ALP-specific activity (a marker of osteoblast differentiation) and between apoptosis and insoluble collagen production (an index of bone-formation activity) in primary osteoblasts of these two mouse strains. It is noteworthy that the variation in apoptosis was achieved by treatments with a number of apoptosis inducers or inhibitors. In addition to their effects on apoptosis, these effectors are known to have various other cellular effects on cell proliferation and/or differentiation. In spite of the potential effects of these effectors on various cellular processes, the inverse relationship between apoptosis and the differentiation status (and bone-formation activity) of these mouse osteoblasts remained evident, indicating that the inverse association between apoptosis and differentiation status and bone-formation activity is probably rather strong. This suggests that apoptosis is likely to be an important regulator of differentiation status and bone-formation activity of these mouse osteoblasts.

Our studies indicate that the difference in osteoblastic apoptosis between the two mouse strains is intrinsic to bone cells. One may wonder how this observation can be compatible with site-specific differences in bone density that have been previously identified. Accordingly, while C3H mice have higher bone density than B6 mice in the long bones, they have lower trabecular bone density in vertebral bone. However, our previous studies of both trabecular and cortical bone sites have shown that, regardless of the measurement sites, bone mineral apposition rate (MAR) is greater in C3H mice than in B6 mice [2, 3]. Other regulatory mechanisms must account for the low trabecular bone density in C3H vertebrae.

The concept of a potential regulatory role for osteoblast apoptosis in bone formation is not entirely new and has been advanced by other investigators [16, 17], particularly Manolagas [17]. Accordingly, Manolagas and coworkers [18] reported that the decrease in bone density, bone formation rate, and MAR in glucocorticoid-treated mice were due to the glucocorticoid-induced apoptosis of osteoblasts. These investigators subsequently provided evidence that the bone-formation action of PTH is also in large part due to an inhibitory action of PTH on osteoblast apoptosis [19]. It has also been reported that smad 3-deficient mice had a lower rate of bone formation and osteopenia, which were associated with dysregulation of osteoblast differentiation and apoptosis [20]. Similarly, it has been reported that sclerostin, an apoptosis inducer, decreased ALP activity in human osteoblast-like cells [21], while ghrelin, an apoptosis inhibitor, increased ALP activity, osteocalcin gene expression, and mineralized nodule formation in MC3T3 cells [22]. However, while these studies raised the possibility that

osteoblast apoptosis may have an important role in the regulation of osteoblast maturation and bone formation, this study, to our knowledge, is the first to document an inverse relationship between apoptosis of osteoblasts and their overall differentiation status and bone-formation activity. More importantly, this study also provides strong circumstantial evidence that basal osteoblast apoptosis can be regulated genetically. We should also emphasize that in the aforementioned studies [18–22] the glucocorticoid, PTH, sclerostin, and ghrelin treatments as well as smad 3 deficiency significantly altered the overall osteoblast population in response to the induced changes in osteoblast apoptosis and the difference in osteoblast apoptosis between C3H and B6 mice did not affect the overall osteoblast population due to compensatory changes in osteoblast proliferation. This indicates that the mechanism for the genetic regulation of osteoblast apoptosis is complicated and may involve compensatory alterations in osteoblast proliferation in such a way that the overall osteoblast population in these inbred mice was not changed.

We should note that the apoptosis difference between the two mouse strains has been reported in cell types other than osteoblast [23, 24]. Thus, it is most likely that the phenotypic difference in cellular activity due to differences in apoptosis between C3H and B6 mice are not limited to bone cells. The inverse correlation between apoptosis and ALP activity (and insoluble collagen production) supports the concept that increased cell differentiation and bone-forming activity can be the consequence of an overall longer life span by decreasing population apoptosis, at least in osteoblasts. Thus, there is a possibility that population apoptosis in other cell types might also have a regulatory role in their maturation and cellular functions.

Regarding the potential pathway(s) responsible for the lower apoptosis in C3H osteoblasts, this study provides strong circumstantial evidence that caspases 3/7 could be involved since there was a corresponding 50% lower caspase 3/7 activity in C3H osteoblasts compared to B6 osteoblasts. In support of this possibility, there is increasing evidence that caspase-3 promotes apoptosis [25, 26] and is required for certain types of cell differentiation [27–30]. However, the regulatory role of caspase-3 in bone formation is controversial. For instance, it was reported that mice with caspase-3 gene deficiency have reductions in MAR, bone formation, and trabecular bone volume [31]. On the other hand, a recent preliminary study reported that mice lacking the caspase-3 gene display increased membranous ossification *in vivo* and calcification *in vitro* [32]. The controversy over the potential regulatory role of caspase-3 in bone formation suggests that this and/or other caspases may have complex roles in the regulation of overall apoptosis and differentiation of bone cells.

The differences between C3H and B6 are primarily due to genetic factors. Previous studies have identified chromosomal quantitative trait loci (QTL) for a number of genes that are responsible for the differences in bone density and bone size between C3H and B6 [33, 34]. Neither caspase-3 nor caspase-7 is located in one of the currently identified QTL. A polar cross-sectional moment-of-inertia QTL has been identified on chromosome 8 [34], but it appears to be distal to the location of the caspase-3 gene. Thus, although our study suggests that the population apoptosis of osteoblasts in this pair of inbred strains is genetically regulated, we conclude that caspases 3 and 7 are not the candidate genes but, rather, could be downstream genes of the candidate genes contributing to the genetic differences in osteoblast apoptosis and bone-formation activity.

While an important limitation of this work is the lack of cause-and-effect evidence that would confirm the contribution of osteoblast apoptosis to the genetic difference in osteoblast differentiation and bone-formation activity between C3H and B6 mice, the significant inverse correlation between apoptosis and cell differentiation and that between apoptosis and bone-forming activity provide a strong rationale for future studies. If our hypothesis that osteoblast apoptosis plays an important regulatory role in osteoblast activity and bone formation is confirmed by future studies, this would open up a new area of research, which may eventually lead to development of novel therapeutic drugs that target osteoblast apoptosis for treatment of bone diseases.

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