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Age-Related Decline in the Osteogenic Potential of Human Bone Marrow Cells Cultured in Three-Dimensional Collagen Sponges

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Abstract Studies with human and animal culture systems indicate that a sub-population of bone marrow stromal cells has the potential to differentiate into osteoblasts. There are conflicting reports on the effects of age on human marrow-derived osteogenic cells. In this study, we used a three dimensional (3D) culture system and quantitative RT-PCR methods to test the hypothesis that the osteogenic potential of human bone marrow stromal cells decreases with age. Marrow was obtained from 39 men aged 37 to 86 years, during the course of total hip arthroplasty. Low-density mononuclear cells were seeded onto 3D collagen sponges and cultured for 3 weeks. Histological sections of sponges were stained for alkaline phosphatase activity and were scored as positive or negative. In the group ≤ 50 years, 7 of 11 samples (63%) were positive, whereas only 5 of 19 (26%) of the samples in the group ≥ 60 years were positive ($p = 0.0504$). As revealed by RT-PCR, there was no expression of alkaline phosphatase or collagen type I mRNA before culture, however there were strong signals after 3 weeks, an indication of osteoblast differentiation in vitro. We performed a quantitative, competitive RT-PCR assay with 8 samples (age range 38–80) and showed that the group ≤ 50 years had 3-fold more mRNA for alkaline phosphatase than the group ≥ 60 years ($p = 0.021$). There was a significant decrease with age ($r = -0.78$, $p = 0.028$). These molecular and histoenzymatic data indicate that the osteogenic potential of human bone marrow cells decreases with age. J. Cell. Biochem. 82: 583–590, 2001. © 2001 Wiley-Liss, Inc.

Key words: bone marrow; aging; osteogenesis; in vitro; three-dimensional

Both in vivo and in vitro animal studies indicate that a sub-population of marrow stromal cells has the potential to differentiate to hard and soft connective tissue cells, including osteoblasts, chondrocytes, and adipocytes [Friedenstein, 1976, Owen, 1985]. More recently, studies with human marrow also indicate that marrow cells can give rise to these different tissues [Beresford, 1989, Long et al., 1990, Vilamitjana-Amedee et al., 1993, Cheng et al., 1994, Gronthos et al., 1994, Rickard et al., 1996, Pittenger et al., 1999]. Culture conditions are important for promoting differentiation of osteoblasts from marrow; standard osteoblastogenic medium contains dexamethasone, β-glycerophosphate, and vitamin C [Leboy et al., 1991, Beresford et al., 1994]. Alkaline phosphatase activity has become a useful index of the early commitment of cells to the osteoblast lineage [Ashton et al., 1980].

Studies with animal bone marrow cells [Perkins et al., 1982, Tsuji et al., 1990, Liang et al., 1992, Roholl et al., 1994, Kahn et al., 1995, Bergman et al., 1996, Frenkel et al., 1997] suggest that there is an age-related decrease in their osteogenic potential, but reports are not consistent for human cells. Shigeno and Ashton found a higher number of proliferative precursor cells in younger than in older subjects [Shigeno and Ashton, 1995]. In a study of

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marrow aspirates from 30 normal subjects, aged 8–80 years, Majors et al. [1997] reported an age-related decline in the number of alkaline phosphatase-positive colonies [Majors et al., 1997]. D'Ippolito et al. reported that in both the presence and absence of dexamethasone, marrow cells from postmortem vertebral bodies showed an age-related decline in formation of alkaline phosphatase-positive colonies [D'Ippolito et al., 1999]. Using the same assay method, Muschler and Boehm reported that there was an age-related decline in alkaline phosphatse-positive colony formation by marrow aspirates that was significant for women but not for men [Muschler and Boehm, 1999]. There are additional reports that conclude that there was no sign of a decline in the osteogenic potential of bone marrow stromal cells with age. Oreffo et al. [1998] found no age-related difference in the colony forming efficiency of human marrow, although there was a significant reduction in the size of the colonies with age [Oreffo et al., 1998a]. Further the same authors reported that there was no effect of aging on alkaline-phosphatase-positive colony forming efficiency in all subjects and that there was little effect of age on colony size in marrow from osteoarthritic subjects [Oreffo et al., 1998b]. Recently, Stenderup et al. concluded that there were no age-related differences in the number and proliferative capacity of human osteogenic cells derived from marrow aspirates from iliac bone [Stenderup et al., 1999].

These disparate conclusions are based on assays for the number of colonies that stain for alkaline phosphatase; these assays are difficult to standardize among individual laboratories. Studies that use colony size or number have been criticized recently because of inappropriate use of parametric statistical methods [Dobson et al., 1999]. Furthermore, monolayer, two-dimensional colony assays have inherent inaccuracies regarding the volume of cells in each colony and problems with necrosis in high density, long-term cultures. The 3D systems may be advantageous, if quantitative data could be obtained, because they enable high density cultivation and provide a geometry similar to that found in vivo. Such 3D systems could then be useful for studying the mechanism of cellular and tissue differentiation. In this study we tested the hypothesis that the osteogenic potential of human bone marrow stromal cells decreases with age using novel 3D collagen sponges that have previously been used for mouse bone marrow cells [Glowacki et al., 1998], chondrocytes [Mizuno and Glowacki, 1996a,b] and murine bone cells [Mueller et al., 1999]. We evaluated development of AlkP-positive cells in 3D collagen sponges by three means. First, we scored histological sections of 3D sponges that were stained for AlkP activity after 3 weeks in vitro. Second, we isolated RNA from five samples before and after 3 weeks culture in 3D collagen sponges and assessed for gene expression of AlkP. Third, with competitive, quantitative RT-PCR we measured the amount of mRNA for AlkP in marrow cultured from young (≤ 50 years) and old (≥ 60 years) subjects.

METHODS

Supplies were obtained from Gibco/BRL (Grand Island, NY) unless otherwise indicated. All media were supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; and L-glutamine 292 μg/ml; Irvine Scientific, (Santa Ana, CA).

Cells

Femoral bone marrow was obtained as discarded material from 39 men (age range 37–80 years) undergoing total hip replacement for osteoarthritis. Subjects were excluded if they were taking medications or had comorbid conditions that could affect skeletal metabolism, including renal insufficiency or rheumatoid arthritis. The study was approved, with annual review, by the BWH Human Research Committee. The low-density mononuclear cells were collected by density centrifugation on Ficoll-Histopaque 1077 (Sigma, St Louis, MO). Yields of cells ranged from 40–800 million.

Three-Dimensional Collagen Sponges

The 3D collagen sponges were made using a method previously reported [Mizuno and Glowacki, 1996a,b]. Briefly, a solution of 0.5% pepsin-digested collagen from bovine skin (Cellagen, ICN Biomedical, Costa Mesa, CA) was neutralized with HEPES and $NaHCO_3$. Two hundred and 50 μl of this collagen solution was poured into a mold and was frozen at $-20^{\circ}C$. After lyophilization, each side of the collagen sponge was exposed to ultraviolet light for 3 h. The dimensions of the dry sponges were 8 μm in diameter and 2 μm in thickness, with a volume of 100 μl.

Three-Dimensional Cell Culture

Forty million low-density mononuclear marrow cells in a volume of 50 μl of medium were seeded on collagen sponges and were completely rehydrated with a subsequent volume of 50 μl. They were cultured at 37°C with 5% CO_2 in α-minimum essential medium (α-MEM), containing 10% FBS and antibiotics. After 24 h, this medium was supplemented with 10 nM dexamethasone (Sigma) [D'Ippolito et al., 1997] and 6 days after seeding, it was supplemented with 5 mM β-glycerophosphate (Sigma) and 170 μM ascorbic phosphate (Wako Chemicals USA, Inc., Richmond, VA). The medium was changed twice a week. Three weeks after seeding, the sponges were harvested and either fixed for histological evaluation or used for RNA extraction and subsequent RT-PCR or quantitative, competitive RT-PCR.

Histological Analysis of Osteoblastic Activity

Sponges were prepared for histological analysis by overnight immersion in 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) and embedment in glycolmethacrylate (JB-4, Polysciences, Warrington, PA). Serial sections were stained for alkaline phosphatase activity (AlkP) with napthol AS-MX phosphate as an index of osteoblast function. Three sections from each AlkP-stained sample were scored as positive or negative, in a masked manner. Because there was similarity in intensity of all positive specimens, it was possible to grade them as either negative or positive, without gradations of positive staining. Samples from 31 subjects (age range: 37–74) were evaluated twice by two investigators.

RNA Isolation

Total RNA was extracted with TRI-Reagent following the manufacturer's instructions. Briefly, the sponges were homogenized with TRI-Reagent/chloroform solution, and the extracted RNA was precipitated with ethanol and isopropanol. The RNA was dissolved in DEPC-treated water and was quantified spectrophotometrically. Before the reverse transcriptase reaction, the RNA was treated with DNAse to remove genomic DNA. Each μg of RNA was treated in a 10 μl solution containing 0.75 U DNAse (Pharmacia Biotech, Piscataway, NJ), 2 mM DTT, 20 mM $MgCl_2$, and 18.5 U RNAse inhibitor (Pharmacia). This solution was incubated for 15 min at 37°C, followed by 70°C for 10 min and subsequent cooling on ice.

cDNA Synthesis

One μg of total RNA and 0.5 μg of oligo(dT) were incubated in a final volume of 12 μl at 70°C for 10 min and chilled on ice for 3 min. 8 μl of 1X reaction solution (final concentration containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.02 M DTT, 9.3 U RNAse inhibitor (Pharmacia), 0.5 mM dNTP mix (Boehringer Mannheim, Indianapolis, IN) and 200 U SuperScriptII-reverse transcriptase) was added and incubated for 60 min at 42°C, followed by 10 min at 72°C to inactivate the enzyme. Each amplification included a control of water and a sample from which reverse transcriptase had been omitted.

PCR Reaction

PCR reactions (50 μl) consisted of PCR buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTP mix (Boehringer Mannheim), 0.4 pmol/ul of 5′ and 3′ primers, and 1 U of Taq DNA polymerase. Samples were amplified with initial denaturation at 94°C for 3 min, followed by 37 cycles of 94°C (30 s), 53°C (2 min), and 72°C (2 min). GAPDH required 35 cycles. A final elongation step at 72°C for 6 min was included. Aliquots (30 μl) of amplified products were separated by agarose gel (2%) electrophoresis in 1X TEA buffer, and DNA fragments were visualized by ethidium bromide staining. The bands were photographed under UV light using instant film (Polaroid, Cambridge, MA).

Oligonucleotides for GAPDH, Alkaline Phosphatase, and Collagen Type I

The quality and equal amount of each cDNA sample was assessed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession # XM 006959.1, bp 601-1052) with primers (sense 5′ ACC ACA GTC CAT GCC ATC AC and antisense 5′ TCC ACC ACC CTG TTG CTG TA) that generated a 451 base pair product (Clontech, Palo Alto, CA). Amplification for alkaline phosphatase (accession # XM 001826.1, bp 970-1325) was performed with primers (sense 5′ CCC AAA GGC TTC TTC TTG and antisense 5′ CTG GTA GTT GTT GTG AGC) that generated a 357 base pair product [Fromigue et al., 1997]. Amplification of collagen type I (accession # XM 004658.1, bp 3279–3739)was

performed with primers (sense 5′ GGA CAC AAT GGA TTG CAA GG and antisense 5′ TAA CCACTG CTC CAC TCT GG) that generated a 461 base pair product [Fromigue et al., 1997].

Quantitative Competitive RT-PCR for Alkaline Phosphatase

The quantitative PCR system is based on the competitive amplification of known amounts of exogenously added cDNA in the same PCR reaction [Wang et al., 1989]. The amount of target cDNA can be determined from the known concentration of the competitor cDNA. To ensure the same efficiency of amplification during the PCR, the competitor is designed to be similar in size (±20%). The competitor cDNA for AlkP was constructed using the "Competitive DNA Construction Kit" (Pan Vera Corp., Madison, WI) following the manufacturer's protocol. Briefly, an oligonucleotide was designed that includes sequences for alkaline phosphatase and the genetic sequence of the template DNA supplied with the kit. With a 30-cycle amplification, the sequences for AlkP were incorporated into a cDNA product with flanking AlkP sequences. This competitor cDNA was purified with SUPREC-02 purification tubes (TAKARA Biomedicals, Osaka, Japan) which eliminates excess primers and dNTP's. The amount of cDNA was determined by spectrophotometric absorption and the predicted size of the competitor cDNA (276 bp) was documented by gel electrophoresis on a 2% agarose gel. Molarity was calculated and a series of 10-fold dilutions of 100 attomoles of competitor cDNA was made with a set of eight reaction tubes containing decreasing concentrations of competitor (100 amol to 0.00001 amol) and a constant amount (104 ng) of target cDNA. This was followed by a subsequent 1:2 dilution series to narrow the range (0.4 amol to 0.0125 amol). For each sample, at least two competitive PCR reactions were performed. Densitometrical analyses were performed with NIH Image 1.61 software, repeated three times. The amount of mRNA was also normalized to the amount of GAPDH in the cDNA. A series of PCR reactions with GAPDH and increasing cycle numbers was performed to assure that 35 cycles was in the linear range (data not shown).

Statistical Analysis

Statistical analysis were performed using the StatView software (Version 4.51; Abacus Concepts, Berkely, CA). The Mann-Whitney nonparametric comparison for unpaired samples was performed to compare the quantity of mRNA expression for alkaline phosphatase of the young and old sets of subjects. Correlation coefficients were determined by the Spearman test for non-Gaussian distributions.

RESULTS

Histological Evaluation

Marrow cells were seeded on the tops of the collagen sponges and became evenly distributed throughout with apparently equivalent cellularity in all samples. Collagen sponges with marrow from young men showed strong positive staining for AlkP throughout the sponge (Fig. 1A). In contrast, sponges seeded with marrow from older subjects did not reveal significant staining greater than background (Fig. 1B). There was little variation apparent in the intensity of positive staining in the 13 positive samples. There were more samples with positive staining in the group ≤ 50 years (7 of 11, 63%) compared to the group ≥ 60 years (5 of 19, 26%). Pearson point-biserial correlation for all samples indicated a significant decrease with age ($P = 0.0504$).

RT-PCR for Alkaline Phosphatase and Collagen Type I

Five samples were used to assess expression of AlkP and Col I mRNA immediately after isolation and after 3 weeks in 3D collagen sponges under conditions known to promote osteoblast differentiation. The marrow was from men whose ages ranged 39–79 years. There was no detectable signal before culture; however strong signals for both genes were found in all samples after three weeks culture (Fig. 2). The absence of expression before culture is taken as evidence that the preparation of low-density cells does not contain osteoblasts or precursor cells with significant elevels of expression of these genes. Their presence after culture indicates that osteoblast-like cells were generated. There were no apparent age-related differences in these qualitative results.

Quantitative RT-PCR for Alkaline Phosphatase

The low-density mononuclear fractions of bone marrow cells from eight patients (age range: 38–80 years) were cultured in collagen

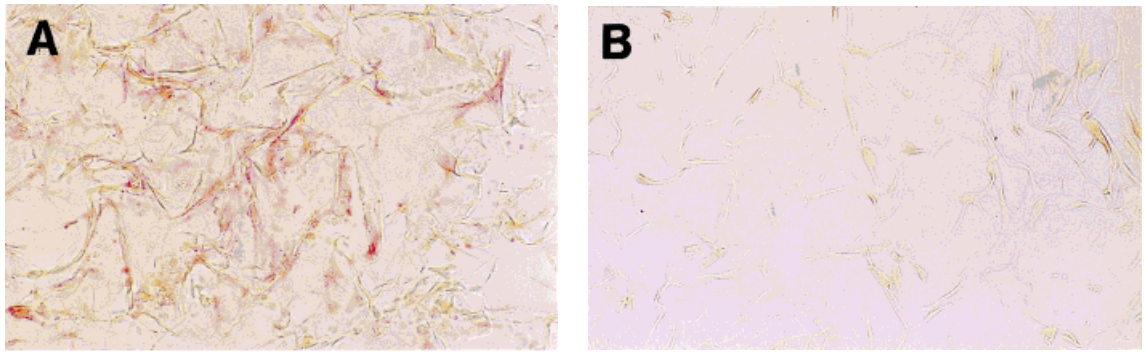

Fig. 1. Photomicrographs of histological sections of 3D collagen sponges stained for alkaline phosphatase activity. **A**: A sponge seeded with marrow from a 38-year-old man shows positive staining. Magnification: 215×. **B**: A sponge seeded with marrow from a 78-year-old man reveals that staining is absent throughout the sponge. Magnification: 215×.

sponges in medium known to promote osteoblast differentiation. After 3 weeks, total RNA was isolated and a quantitative, competitive RT-PCR was performed for expression of the AlkP gene (Fig. 3). The age-dependent decrease in number of AlkP mRNA transcripts was statistically significant (Fig. 4A; $r = 0.78$, $p = 0.028$). The data were also analyzed in two groups of younger and older subjects. The group ≤ 50 years expressed twice the number of mRNA transcripts for AlkP compared to the group ≥ 60 years, either when expressed as absolute number of transcripts (Fig. 4B; $p = 0.029$) or number of transcripts relative to the amount of the housekeeping gene, GAPDH (Fig. 4C; $p = 0.029$).

DISCUSSION

These studies show that there is an age-dependent decrease in AlkP activity and in AlkP transcript number in normal human bone marrow cells cultured in 3D collagen sponges. Most information concerning skeletal progenitors/precursors in marrow has been obtained from monolayer cultures in which bone nodules develop with time. Aubin described at least two stages of osteoprogenitor cells in rat marrow: those that do and those that do not require glucocorticoids to differentiate [Aubin, 1999]. The use of 3D collagen sponges has advantages to characterize matrix components [Mueller et al., 1999] and other 3D systems have been used to some extent for human marrow [Bruder et al., 1998, Fricain et al., 1998, Oreffo et al., 1998c]. The 3D collagen sponge system used in these studies attempts to model the geometry of bone marrow and allows for high density culture. It enabled molecular analysis of osteoblast differentiation and reveals an age-related decrease in osteoblast differentiation of human bone marrow cells. These collagen sponges have been used to show another age-related differ-

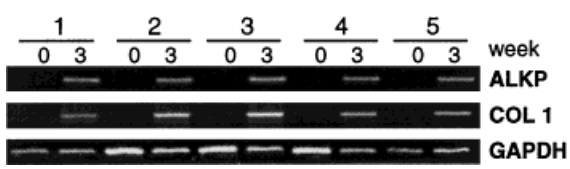

Fig. 2. Expression of alkaline phosphatase (ALKP), collagen type I (COL I), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 5 samples of human marrow cells before and after 3 weeks culture in 3D collagen sponges. Equal volumes of cDNA were amplified with gene-specific primer pairs and resulting PCR products were separated by electrophoresis on 2% agarose and visualized by ethidium bromide staining. Patients ages were 1: 39Y; 2: 46Y; 3: 70Y; 4: 72Y; 5: 79Y.

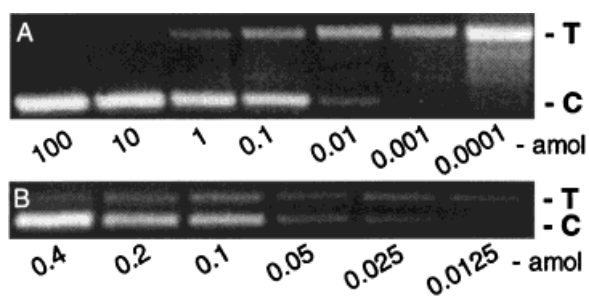

Fig. 3. Agarose electrophoresis of a dilution series of competitive RT-PCR in which the alkaline phosphatase competitor (C) was added to a constant amount (104 ng) of target cDNA (T). Agarose gels (**A**) show a 1:10 dilution series and (**B**) a 1:2 dilution series between 100 and 0.00001 attomoles of competitor. In this example with marrow from a 72-year old subject, the ratio of target and competitor appears to be the same at 0.05 amol competitor.

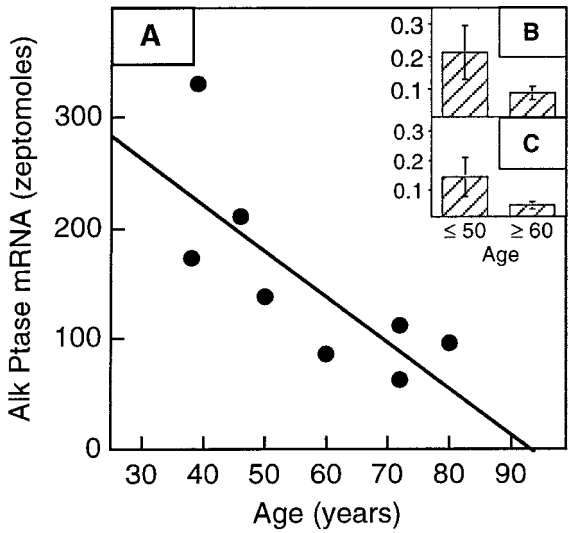

Fig. 4. Effect of age on number of transcripts for alkaline phosphatase (AlkP). (**A**) Transcript number (zeptomoles/μg RNA) decreased with age ($r = 0.78$, $p = 0.028$). Transcripts are also expressed for the means (±standard deviation) for the two age groups as (**B**) attomoles of mRNA for AlkP per μg RNA and (**C**) attomoles of mRNA for AlkP relative to the amount of GAPDH.

ence in human marrow cell biology. Expression of osteoprotegerin mRNA was found to be 5-fold greater in marrow from 9 women younger than 65 years than in marrow from 9 subjects older than 65 years [Makhluf et al., 2000]. Those results are consistent with the hypothesis that bone marrow cells may contribute to age-related bone loss by both catabolic and anabolic mechanisms. Osteoclastogenesis may increase with age in part because of the decline in expression of osteoprotegerin, a mediator that blocks osteoclast differentiation. The data presented herein indicate that with age, the potential of marrow stromal cells to differentiate to osteoblasts may decrease. Thus bone loss could result from the growing preponderance of osteoclasts relative to osteoblasts.

These data are consistent with reports of age-related declines in development of AlkP-positive colonies from human marrow cells. [Nishida et al., 1999] investigated the effect of age on colony-forming units-fibroblastic (CFU-Fs) of human bone marrow cells from subjects of different ages. The authors found that the levels of AlkP-positive CFU-Fs declined sharply after 10 years of age, with those above 20 years exhibiting a lower number of AlkP-positive CFU-Fs. They concluded that this change in AlkP-activity may be due to a decreased number of osteoprogenitor cells differentiating into osteoblasts. Assays for colonies that stain for AlkP cytochemical activity are commonly used to enumerate cells with osteogenic potential, but not all reports show similar results. Colony assays have been reported to show an inverse relationship with age [Shigeno and Ashton, 1995, Majors et al., 1997, D'Ippolito et al., 1999, Muschler and Boehm, 1999] or no relationship with age [Oreffo et al., 1998a,b, Stenderup et al., 1999]. Part of this apparent inconsistency may be due to methodological differences (particularly regarding different mitogens in lots of serum), insufficient power to detect differences, or to inappropriate statistical tests [Dobson et al., 1999]. There may also be differences in the starting populations of cells, with different studies using intraoperative material, marrow aspirates from iliac crest, or cadaveric vertebral bone particles. Most studies with human marrow-derived cells require glucocorticoids for osteoblast differentiation, although D'Ippolito et al. detected large numbers of AlkP-positive colonies in cells from vertebral marrow cultured without dexamethasone, especially in subjects less than 30 years of age [D'Ippolito et al., 1999]. In that study, the magnitude of stimulation by dexamethasone was minimal and there was a significant decrease in the size of the colonies in the presence of dexamethasone. Whether those properties are unique to vertebral marrow, to aspects of the cell isolation technique that involved production of bone chips, or to the fact that all subjects had fatal traumatic injuries cannot be determined at this time. It is possible that their study and perhaps others may have employed more differentiated (e.g., glucocorticoid-independent) starting cells than used in other studies.

Cell sorting techniques have been useful to enrich for human osteogenic lineage cells [Chen et al., 1997, Joyner et al., 1997], especially those using the monoclonal antibody STRO-1 [Gronthos et al., 1994, Oyajobi et al., 1999]. Clonogenic progenitors and cell lines have also been useful for studying regulation of human osteoblast differentiation [Hicok et al., 1998; Prabhakar et al., 1998; Oyajobi et al., 1999; Pittenger et al., 1999]. Primary cultures are less convenient for comprehensive analysis but they provide the most direct approach for testing for age effects. Marrow discarded in the course of total hip replacement was used in this

report. Marrow from subjects requiring surgery for non-inflammatory osteoarthritis was studied. The low-density fraction of marrow contains relatively undifferentiated cells with a high nuclear-to-cytoplasmic ratio and is largely comprised of hematopoietic stem cells with a smaller proportion of adherent, or stromal cells. The absence of AlkP or collagen I mRNA in the starting preparations used herein suggest their primitive nature. Previous reports of monolayer studies with marrow from similar subjects indicated that in vitro secretion of Interleukin-6 and -11 was increased with age and was lower in marrow from subjects receiving estrogen replacement therapy (7.5%) than from age-matched controls [Cheleuitte et al., 1998], that constitutive secretion of Insulin-like Growth Factor Binding Protein-3 was increased with age [Rosen et al., 1997], and that a population of marrow stromal cells express an extracellular calcium-sensing receptor [House et al., 1997]. This source of human marrow has thus been shown to produce osteotrophic mediators and to be a source of bone cells.

Current methods do not indicate whether there are changes in the number of cells with capacity to proliferate and differentiate to osteoblasts; in the expansion potential of such cells; or in the synthetic activity of osteoblasts that differentiate from older marrow. Thus, there remain questions about the relative importance of proliferative and differentiation potentials of skeletal progenitors, especially with respect to age-related changes. New molecular tools may be needed to answer them. Rigorous answers to these fundamental questions may require development of automated high-throughput instrumentation. Clearly, more information is needed on the interconversion of musculoskeletal cell lineages [Lecoeur and Ouhayoun, 1997; Park et al., 1999; Pittenger et al., 1999] as a function of age and on the potential of anabolic factors to rejuvenate aging marrow.

In conclusion, this investigation used a 3D culture system with histoenzymatic and quantitative RT-PCR assays and supports the conclusion that there is an age-related decline in the osteogenic potential in human marrow.

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