The STRO-1+ Fraction of Adult Human Bone Marrow Contains the Osteogenic Precursors

By S. Gronthos, S.E. Graves, S. Ohta, and P.J. Simmons

The monoclonal antibody STRO-1 identifies clonogenic bone marrow stromal cell progenitors (fibroblast colony-forming units [CFU-F]) in adult human bone marrow. These STRO-1+ CFU-F have previously been shown to give rise to cells with the phenotype of fibroblasts, adipocytes, and smooth muscle cells. In this study, the osteogenic potential of CFU-F derived from the STRO-1+ fraction of adult human bone marrow was determined. CFU-F were isolated from normal bone marrow aspirates by fluorescence activated cell sorting, based on their expression of the STRO-1 antigen. Osteogenic differentiation was assessed by the induction of alkaline phosphatase expression, by the formation of a mineralized matrix (hydroxyapatite), and by the production of the bone-specific protein osteocalcin. STRO-1+ cells were cultured in the presence of dexamethasone (DEX; 10^-7 mol/L), ascorbic acid 2-phosphate (ASC-2P; 100 μmol/L), and inorganic phosphate (PO_4^3-; 2.9 mmol/L). After 2 weeks of culture, greater than 90% of the cells in each CFU-F colony stained positive for alkaline phosphatase using a monoclonal antibody specific for bone and liver alkaline phosphatase. Alkaline phosphatase activity was confirmed by histochemistry. A mineralized matrix developed in the CFU-F cultures, after 4 weeks of culture in the presence of DEX, ASC-2P, and PO_4^3-. Mineralization was confirmed by both light and electron microscopy. The mineral was identified as hydroxyapatite by electron dispersive x-ray microanalysis and by x-ray diffraction analysis. In replicate cultures, osteocalcin release was shown after exposure of the cells to 1,25-dihydroxyvitamin D_3 (10^-7 mol/L) both by radioimmunoassay and Northern blot analysis. This work provides direct evidence that adult human bone marrow-derived CFU-F are capable of differentiating into functional osteoblasts and that osteoprogenitors are present in the STRO-1+ population.

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In vivo, hematopoietic cells develop in intimate contiguity with a phenotypically and probably functionally diverse population of mesenchymal, connective tissue type cells that, collectively, represent the stromal tissue of the bone marrow (BM). A considerable body of evidence derived from studies in vivo and in vitro shows that the hematopoietic microenvironment (HM) of the hematopoietic organs is mediated in large part by this heterogeneous population of stromal cells that endow these organs with the unique capacity to support hematopoietic cell development.

Cell types comprising the stromal tissue of the BM include vascular endothelial cells, smooth muscle cells, reticular cells, adipocytes, and osteoblasts. This diversity of stromal elements has complicated attempts to elucidate the role of each cellular component in the support of hematopoiesis. Moreover, the origin and developmental relationship between each component of the marrow stroma remains to be determined. Studies in rodents and in humans clearly show that marrow stromal tissue is capable of extensive regeneration after a variety of insults ranging from radiation to mechanical disruption. These data raise the question as to whether each of the various stromal cell types has proliferative potential and therefore represent self-maintaining populations within the BM. Alternatively, does there exist a population of stromal cell precursors with the capacity to give rise to each of the aforementioned stromal elements and is thus responsible for the turnover of marrow stromal tissue under steady-state and perturbed situations?

Putative stromal cell precursors have been identified in a number of species, including humans, by their ability to form colonies of cells morphologically resembling fibroblasts when single-cell suspensions of BM mononuclear cells are explanted at appropriate densities in liquid culture. The clonogenic progenitor responsible for colony formation under these conditions is referred to as fibroblast colony-forming unit (CFU-F). After their initial description by Friedenstein, subsequent studies of the CFU-F population in the murine system showed heterogeneous developmental potentials after ectopic transplantation of individual CFU-F colonies. A minor proportion of CFU-F were found to develop into marrow organs containing the full spectrum of stromal cell types, including bone cells, whereas the remainder of colonies generated stromal organs comprising only bone cells or soft connective tissue. Based on these and other data, Owen proposed the stromal stem cell hypothesis in which, by analogy with the hematopoietic system, there exists a hierarchy of cellular differentiation supported at its apex by a small compartment of self-renewing, multipotent stromal stem cells.

Although formal proof of the existence of stromal stem cells is lacking, this hypothesis has nevertheless provided a useful conceptual basis from which to investigate the developmental potential of marrow stromal tissue. The work of Friedenstein was the first to show the osteogenic potential in the mouse. Subsequent in vitro studies have shown the presence of cells with the phenotype of bone cells in both rat and mouse BM cultures when cultured under conditions permissive for osteogenic development (L-ascorbate, dexamethasone, and β-glycerol-phosphate). More recently, a cloned murine cell line from the marrow stroma was established that displays osteogenic characteristics, providing direct evidence that a single stromal progenitor can differenti-
ate into osteoblast-like cells. In human studies, the ectopic transplantation of BM cells in diffusion chambers in nude mice has failed to provide evidence of the existence of cells with osteogenic potential in normal adult human marrow. However, the formation of bone and cartilage in diffusion chambers containing marrow from young children has been reported. More recently, cultured adult human BM cells placed into ceramic cubes have been shown to form bone (but not cartilage) when implanted in nude mice. Thus far, the use of in vivo assays to measure the osteogenic potential of human marrow cells is clearly still at a developmental stage. Other studies have examined the osteogenic potential of cultured human BM stromal cells by the in vitro induction and detection of characteristic bone cell markers, including collagen type I synthesis, alkaline phosphatase expression, osteocalcin production, and bone mineral formation. Collectively, these studies show the presence of cells with an osteoblast-like phenotype in human BM cultures. However, given the heterogeneity of the stromal cell population in whole BM, the question arises as to whether these cells are already committed bone cells and whether the osteogenic phenotype can be induced in a more primitive stromal cell precursor population.

In the present study, we examine the possibility of establishing a reproducible and defined in vitro culture system to directly show the osteogenic potential of CFU-F isolated from adult human BM. To avoid the culture of whole BM that could contain cells already committed to the osteogenic lineage, human BM CFU-F were purified using the mouse monoclonal antibody (MoAb) STRO-1. BM mononuclear cells sorted on the basis of STRO-1 expression are capable of establishing an adherent stromal layer in vitro, consisting of a number of phenotypically distinct stromal cell types, including fibroblasts, smooth muscle cells, and adipocytes.

We show here that in all BM samples examined, the MoAb STRO-1 identifies cells with osteogenic potential as assessed by the expression of molecules of cells that exhibit three independent markers of differentiated bone cells: alkaline phosphatase expression; 1,25-dihyroxyvitamin D$_2$-dependent induction of the bone-specific protein, osteocalcin; and production of a mineralized matrix (hydroxyapatite).

**MATERIALS AND METHODS**

Subjects. Fresh BM aspirates were obtained (after obtaining informed consent) from the iliac crest and/or the sternum of normal adult volunteers, according to procedures approved by the ethics committee at the Royal Adelaide Hospital. BM mononuclear cells (BMMNCs) were obtained by centrifugation over Picoll (1.077 g/mL; Lymphoprep; Nycomed, Oslo, Norway) at 400g for 30 minutes. The BMMNCs were washed twice in Hanks' Buffered Saline Solution (HBSS) supplemented with 5% fetal calf serum (FCS; batch 593; Gibco BRL, Victoria, Australia) and 10 mmol/L HEPES, pH 7.35 (GIBCO-BRL) (HHF), before immunolabeling and flow cytometry.

Bone chips from the tibia were obtained during routine knee replacements from the Department of Orthopaedic Surgery and Trauma at the Royal Adelaide Hospital. Explants of trabecular bone were cultured as described previously. Cells growing out of the explants display a characteristic osteoblastic phenotype in culture. Primary human bone cells were used as a positive control for osteocalcin mRNA.

**Fluorescence-activated cell sorting (FACS).** This procedure has been reported previously. Briefly, BMMNCs (1 to 3 x 10$^6$ cells) were pelleted in 5 mL polypropylene tubes (Falcon; Becton Dickinson, Linkon Park, NJ) and resuspended in 200 mL of saturating concentrations of the mouse IgM MoAb STRO-1 for 45 minutes at 4°C or with an isotype-matched MoAb of irrelevant specificity, 1A6.12 (donated by Dr L.K. Ashman, Department of Hematology, IMVS, Adelaide, Australia). The cells were then washed twice in HHF at 4°C, before the addition of 200 mL of a 1/50 dilution of phycoerythrin (PE)-conjugated goat antimouse IgM m- chain–specific (Southern Biotechnology Associates, Birmingham, AL). The cells were incubated for 45 minutes at 4°C and then washed twice in HHF before being sorted using a FACSStaHL flow cytometer (Becton Dickinson, Sunnyvale, CA). Cells were maintained at 4°C throughout the cell sorting procedures. STRO-1+ cells were defined as those exhibiting a level of fluorescence greater than 95% of that obtained with the isotype-matched control antibody, 1A6.12.

**In vitro formation of bone.** This method is a modification of two different procedures, adapted for human BM cells. STRO-1+ cells (2 x 10$^6$ T-25 culture flask) were cultured in a modification of Eagle's medium (α-MEM; Flow Laboratories, Irvine, Scotland) supplemented with 20% FCS, L-glutamine (2 mmol/L), β-mercaptoethanol (5 x 10$^{-5}$ mol/L) and with or without L-ascorbic acid 2-phosphate (100 μmol/L; ASC-2P; Novachem, Melbourne, Australia) at 37°C in 5% CO$_2$. After 7 days, the culture medium was switched to α-MEM supplemented with 10% FCS, L-glutamine (2 mmol/L), dexamethasone sodium phosphate (DEX; 10 μmol/L; David Bull Laboratories, Sydney, Australia), ASC-2P (100 μmol/L), KH$_2$PO$_4$ (1.8 mmol/L; BDH Chemicals, Poole, UK) to give a final phosphate concentration of 2.9 mmol/L, and HEPES (20 mmol/L) for those cultures initiated with ASC-2P. Control cultures without ASC-2P were maintained in α-MEM supplemented with 10% FCS and L-glutamine (2 mmol/L). The media was changed twice a week for varying periods up to 6 weeks.

**Alkaline phosphatase activity.** Two-week-old BM cultures grown under osteogenic inductive conditions (ascorbic acid 2-phosphate [ASC-2P], DEX, and inorganic phosphate [PO$_4$] and control cultures, were incubated with either a mouse MoAb specific for human liver and bone alkaline phosphatase, B4-78 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), for 45 minutes at 4°C. Nonspecific staining was assessed by incubating cells under identical conditions with an isotype-matched MoAb of irrelevant specificity. 3D3 (generously provided by Dr L.K. Ashman). Cultures were then washed twice in RPMI before being fixed in 2% paraformaldehyde for 20 minutes at 4°C. After fixation, a Vectastain ABC kit for mouse IgG (PK-4010; Vector Laboratories, Burlingame, CA) and a peroxidase substrate kit AEC (SK-4200; Vector Laboratories) were used to localize specifically bound antibody according to the manufacturer's recommendations. Alkaline phosphatase activity was confirmed by histochemistry performed on replicate cultures using a Sigma (St Louis, MO) in vitro diagnostic kit, 85L-2, according to the manufacturer's recommendations. After immunostaining and histochemical staining, the cells were counter stained with Mayer's hematoxylin and examined and photographed using an Olympus IMT-2 inverted light microscope (Olympus Optical, Ltd, Tokyo, Japan). Photographs were taken using Kodak Technical PAN black and white film (Eastman Kodak, Rochester, NY).

**Von Kossa staining of bone-derived mineral.** Mineralized BM cultures and controls were gently teased off as single layers using a plastic pipette. The cell layers were fixed in 95% ethanol for 60 minutes before being infiltrated and embedded in 50% glycol methacrylate (Tokyo Kasei, Tokyo, Japan) and 50% methyl methacrylate (RHD Chemicals) over 1 week at room temperature. Transverse sections (5 μm) of the bone layers were cut using a glass knife on a Jang K motorized sledge microtome (Reichert-Jung, Heidelberg, Germany). Von Kossa staining was performed according to the
Sections were washed twice in distilled water and then stained in 5% aqueous AgNO₃ (May & Baker Laboratory Products, West Footscray, Victoria, Australia) for 60 minutes under UV light. After staining with AgNO₃, the sections were placed in 5% sodium thiosulphate (BDH Chemicals) for 1 minute. The sections were counterstained with Mayer’s haematoxylin and eosin and mounted in Univar aqueous mountant (BDH Chemicals). Von Kossa staining was analyzed using an Olympus SZ-PT light microscope.

Detection of osteocalcin by radioimmunoassay (RIA). This method is a modification of that described by Lajunen et al and was adapted for serum-deprived conditions. Six-week-old mineralized BM cultures and controls were washed twice in phosphate-buffered saline (PBS) and then switched to serum-deprived medium as previously described, supplemented with CaCl₂-2H₂O (2 mmol/L; Sigma), KH₂PO₄ (1.8 mmol/L), menadione sodium bisulphite (10⁻⁴ mol/L; vitamin K₃; Sigma), and calcitriol (10⁻⁷ mol/L; 1,25-dihydroxyvitamin D₃; donated by F. Hoffmann-La Roche Ltd, Basel, Switzerland) for 48 hours at 37°C in 5% CO₂. After incubation, the media was removed and the osteocalcin concentration in the medium was measured by a specific RIA (Department of Endocrinology of the IMVS, Adelaide, South Australia) using an antibody raised against bovine osteocalcin (>90% homologous to human osteocalcin). In preliminary experiments, media supplemented with 1% FCS contained approximately 3 ng of osteocalcin per milliliter, in contrast to serum-deprived medium, which contained less than the detection limit of the assay of 0.4 ng of osteocalcin per milliliter. Background levels of osteocalcin detected in serum-deprived medium were compared with the osteocalcin levels detected in the cultures (t-test). The results were expressed as the mean values from duplicate cultures of nanograms of osteocalcin per 10⁶ cells per 48 hours of induction with 1,25-dihydroxyvitamin D₃.

Detection of osteocalcin mRNA by Northern blot analysis. Total RNA from the same BM cultures used for RIA and from primary human bone cultures was extracted by the acid guanidium-phenol-chloroform method as described previously. Denatured total RNA (20 μg) was separated by 1% agarose gel electrophoresis and transferred to a nylon membrane (Shleicher & Schuell, Dassel, Germany). After UV cross-linking and prehybridization, the blots were hybridized in 50% formamide to a synthetic oligonucleotide probe for human osteocalcin (5′-CCACTCGTCACAGTCCGGATTGAGCTCACAACACTCCCT-3′), complementary to the mRNA sequence coding for amino acids 20-32 of mature osteocalcin as previously described. The probe was end-labeled with (γ-³²P)-ATP (Bresatec, Adelaide, Australia). Washes were performed in 2x sodium saline citrate (SSC) at room temperature, followed by washings at 42°C for 1 hour. The membrane was exposed to Kodak X-Omat film at −70°C for 72 hours with a Cronex intensifying screen (du Pont, Wilington, DE). The integrity of the RNA analyzed was confirmed by ethidium bromide staining.

Transmission electron microscopy (TEM). Six-week-old mineralized BM cultures were washed in 0.05 mol/L sodium cacodylate buffer and then fixed in 2.5% glutaraldehyde (EM grade) in 0.05 mol/L sodium cacodylate buffer for 2 hours at room temperature. After washing with 0.05 mol/L cacodylate buffer, the cultures were postfixixed with 2% osmium tetroxide (VIII; BDH Chemicals) in cacodylate buffer for 1 hour at room temperature and then washed in cacodylate buffer. After this procedure, the cultures were dehydrated with graded alcohol (70%, 90%, and 100% ethanol solutions) for two 15-minute changes for each alcohol grade. Epoxy resin (TAAB Laboratories, Berkshire, UK) was then used to infiltrate the cultures overnight at 37°C. Polymerization of the resin was performed at 60°C for 24 hours under vacuum. Ultrathin sections were cut on a LKB 8800 Ultrrotome II (Bromma, Sweden) and mounted on copper grids. Sections were then examined using a JEOL 1200 EX II
OSSEOGENIC POTENTIAL OF STRO-1\(^+\) BM CFU-F

Fig 2. The mineral formed by human BM CFU-F cultured for 4 weeks in the presence of ASC-2P, DEX, and PO\(_4\)\(^-\) was shown to be Von Kossa positive (original magnification x100) (A). Cultures were counter-stained with hematoxylin. A light microscopic view of the adherent layers of replicate cultures shows the development of mineral deposits (large arrow) and the appearance of clusters of adipocytes (small arrow) (original magnification x200) (B). Deposits of mineral were never observed in the BM CFU-F cultures grown in the presence of FCS alone (original magnification x200) (C).

Tokyo, Japan) transmission electron microscope. Photographs were taken using ILFORD EM Technical film (ILFORD Pty Ltd, Mobberley, UK). Mineral deposits were analyzed using a Tracor-Northern Series II EDX System (EDX: Energy Dispersive x-ray analyzer). Electron diffraction analysis was performed using a camera length of 100 cm on the mineralized bone cultures and on a commercially available hydroxyapatite standard (hydroxyapatite type 1; Sigma).

RESULTS

Induction of alkaline phosphatase expression on BM CFU-F. STRO-1\(^+\) and STRO-1\(^-\) cells derived from BMMNCs from 3 different individuals were isolated by FACS and cultured under standard CFU-F conditions, in the presence or absence of ASC-2P. All cultures derived from STRO-1\(^-\) cells failed to produce any CFU-F after 2 weeks in culture, in accord with previous observations,\(^3\) and were therefore discarded. In the STRO-1\(^+\) cultures, the CFU-F were allowed to develop for 7 days, at which time the cultures were supplemented with a combination of ASC-2P, DEX, and PO\(_4\)\(^-\) for those cultures initiated with ASC-2P. The addition of DEX at the start of culture caused a marked decrease in the proliferation of CFU-F that was evident by day 7 of culture. However, delayed addition of DEX did not result in the inhibition of stromal cell proliferation (data not shown). For this reason, the addition of DEX was therefore performed 7 days after culture initiation.

BM CFU-F colonies were analyzed for their expression of alkaline phosphatase, a well-documented maker of bone cell differentiation, by immunoperoxidase staining using the MoAb B4-78, which identifies the bone- and liver-specific form of the enzyme.\(^4\) (Fig 1). Alkaline phosphatase activity was confirmed by histochemistry performed on replicate cultures (data not shown). After 2 weeks of culture, all of the CFU-F colonies grown in the presence of ASC-2P, DEX, and PO\(_4\)\(^-\) were found to express alkaline phosphatase (Fig 1B). Moreover, within individual colonies, consistently greater than 90% of the cells exhibited alkaline phosphatase. In contrast, the CFU-F cultured in the presence of FCS alone showed a heterogeneous staining pattern, with typically \(\leq\)25% of the cells in each colony showing positivity for alkaline phosphatase (Fig 1A). To examine which factor was responsible for inducing alkaline phosphatase activity, STRO-1\(^+\) cells were grown in the presence of ASC-2P, DEX, and PO\(_4\)\(^-\) and in multiple combinations of the three. DEX was found to be responsible for the increased expression of alkaline phosphatase on CFU-F colonies in all groups examined when compared with CFU-F grown in FCS alone (data not shown).

STRO-1\(^+\) BMMNCs can be induced to form mineral. The adherent layers of BM cultures after 4 weeks in osteogenic growth conditions (ASC-2P, DEX, and PO\(_4\)\(^-\)) displayed large areas of mineralized material that stained positively with the Von Kossa technique (Fig 2A). Adipocytes were also observed in the same cultures throughout the adherent layers, as seen by light microscopy under phase contrast (Fig 2B). Mineral deposits were not found in the control cultures with FCS alone (Fig 2C).
A view of the whole culture flasks under dark field illumination shows the extent of mineralization after 6 weeks of induction with ASC-2P, DEX, and PO4 (Fig 3A). Transverse sections of the adherent layers from 6-week-old cultures showed an extensive multilayered growth pattern that was subsequently confirmed by electron microscopic examination (see Fig 5 below). A similar pattern of growth was seen in cultures grown in FCS alone (Fig 4B). However, mineralization was only evident in cultures supplemented with ASC-2P, DEX, and PO4, as shown by positive staining with the Von Kossa reaction (Fig 4A). Human foreskin fibroblasts cultured under identical conditions for 6 weeks failed to produce a mineralized matrix (data not shown).

Ultrastructural examination of the cultures by TEM showed that the mineral was associated with collagen fibrils in the extracellular matrix of the adherent layers (Fig 5A and B). EDX was used to evaluate the nature of the mineral in these cultures. The calcium to phosphorus (Ca/P) ratio in the mineralized cultures was 1.76 ± 0.10 (n = 3; Fig 5C), which was consistent with the Ca/P ratio of hydroxyapatite in vivo (1.67). X-ray diffraction studies also showed that the crystal structure of the mineral deposits in the induced cultures (ASC-2P, DEX, and PO4) was identical to the crystal diffraction patterns obtained from the hydroxyapatite standard (Fig 5D).

To determine the incidence of CFU-F with osteogenic potential, we isolated 21 CFU-F clones by limiting dilution in standard CFU-F growth conditions. Day-7 colonies were subsequently cultured for 4 weeks in the presence of DEX, ASC-2P, and PO4. After this procedure, the colonies were examined for the formation of a mineralized matrix. All of the 21 CFU-F clones cultured in the presence of DEX, ASC-2P, and PO4 were shown to be positive for Von Kossa staining. In addition, adipocytic formation was observed in only a proportion (48%) of the colonies examined (data not shown).

Osteocalcin production by BM CFU-F. Six-week-old mineralized BM cultures and controls were washed in PBS and then placed under serum-deprived conditions. The biosynthesis of osteocalcin was stimulated for 48 hours by the addition of 1,25-dihydroxyvitamin D3 (1,25-Vit D3). Samples were then taken from the medium of all the cultures and analyzed for the presence of osteocalcin by RIA. Varying levels of osteocalcin (2.6 to 19.8 ng/10^6 cells/48 hours 1,25-Vit D3) were detected in the mineralized BM cultures, but not in control cultures grown in the presence of FCS.
Fig 4. Von Kossa staining of transverse sections of the adherent layers from cultured BM CFU-F grown for 6 weeks in the presence of ASC-2P, DEX, and PO4 (original magnification ×200) (A) or in the presence of FCS alone (original magnification ×200) (B). The sections were counter-stained with hematoxylin and eosin. Areas of Von Kossa-positive mineral deposits (arrow) were observed throughout the adherent layers of the cultures treated with ASC-2P, DEX, and PO4 (A). No Von Kossa-positive mineral could be detected in the adherent layers of the control cultures treated with FCS alone (B).
of bone cell development has recently been questioned. In human studies, DEX has been shown to be required for the differentiation of osteoblast-like cells present in BM stromal cultures. In vitro, DEX was shown to inhibit the 1,25-Vit D₃-induced expression of osteocalcin in human BM cells and human bone cells. In contrast, DEX has been found to increase the expression of mRNA for many genes, including those encoding for alkaline phosphatase and various proto-oncogenes, such as c-fos, that are associated with osteogenic differentiation. In the present study, the addition of DEX to the culture medium increased the expression of alkaline phosphatase on CFU-F colonies.
OSTEOGENIC POTENTIAL OF STRO-1⁺ BM CFU-F

Table 1. Measurement of Osteocalcin Release in Human BM CFU-F Cultures

<table>
<thead>
<tr>
<th>BM Sample</th>
<th>Control (NS)</th>
<th>ASC-2P + DEX + PO₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3 ± 0.3</td>
<td>19.8 ± 6.9</td>
</tr>
<tr>
<td>2</td>
<td>0.2 ± 0.2</td>
<td>9.7 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>0.8 ± 0.6</td>
<td>2.6 ± 1.1</td>
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</table>

Values are the mean ± SE nanograms of osteocalcin per 10⁶ cells per 48 hours of 1,25-Vit D₃ treatment. Adult human CFU-F cultures were analyzed for the presence of osteocalcin by RIA. Osteocalcin levels in each sample were compared with the background levels of osteocalcin detected in the serum-deprived medium alone. Significant levels of osteocalcin were only detected in those cultures treated with ASC-2P, DEX, and PO₄⁺.

Abbreviation: NS, not significant.

* P < .05 (t-test).

Synthesis by bone cells in vitro of a mineralized matrix has previously been shown to be dependent on supplementation of their growth medium with a source of inorganic phosphate. In this study, inorganic phosphate was used in place of β-glycerophosphate, which is a nonphysiological organic phosphate substrate of alkaline phosphatase. The presence of β-glycerophosphate in animal studies has been shown to cause ectopic mineralization in cultures of fetal rat parietal cells and skin fibroblasts, which does not occur using inorganic phosphate. In parallel studies, human foreskin fibroblasts cultured in the presence of ASC-2P, DEX, and PO₄⁺ for a period of 6 weeks were found to be Von Kossa negative.

Purified STRO-1⁺ human BM cells were cultured under standard CFU-F growth conditions in the presence of ASC-2P. Day-7 CFU-F cultures were then switched to osteogenic inductive conditions (ASC-2P, PO₄⁺, and DEX). After 2 weeks, greater than 90% of the cells in all of the CFU-F colonies were shown to express alkaline phosphatase. Previous studies have shown that alkaline phosphatase is involved with the induction of hydroxyapatite deposition in collagen matrices in vivo. The results of the present study showed that the induction of alkaline phosphatase expression preceded the appearance of Von Kossa-positive mineral by 7 to 14 days in the induced cultures. TEM analysis of the adherent layers showed the presence of deposits of hydroxyapatite-like crystals in association with the collagen fibrils in the matrix. EDX analysis showed that the mineral was composed of calcium (Ca) and phosphorus (P), consistent with the Ca/P ratio of hydroxyapatite crystals in vivo. In addition, the crystal structure of the mineral was found to be identical to the crystal structure of the hydroxyapatite standard, as determined by x-ray diffraction analysis. The presence of mineral deposits, which stained positive for the Von Kossa reaction, was shown in all CFU-F colonies isolated by limiting dilution. This finding is in contrast to the findings in studies of rodent BM that reported that only a proportion of CFU-F were capable of developing an osteogenic phenotype. This discrepancy may be attributed to the presence of accessory cells in the relatively crude BM preparations used in these studies, which may affect the expression of the osteogenic phenotype of CFU-F in vitro. In addition, the absence of DEX and ascorbate in the culture medium may lead to an underestimation of the incidence of osteogenic precursors in rodent BM.

Osteocalcin is an osteoblast-specific protein and was therefore used in this study as a marker of osteogenic differentiation. Cultured primary human bone cells have been shown to synthesize osteocalcin in the presence of 1,25-Vit D₃. In the present study, comparable levels of osteocalcin were found in the culture medium of human BM cultures induced for 6 weeks under osteogenic conditions (ASC-2P, DEX, and PO₄⁺) and stimulated with 1,25-Vit D₃ for 48 hours. The 1,25-Vit D₃-stimulated induction of osteocalcin mRNA was confirmed by Northern blot analysis.

This work provides direct evidence that purified human BM CFU-F under defined in vitro culture conditions are capable of osteogenic differentiation. These findings also indicate that the osteogenic precursors are found in the STRO-1⁺ population of human BM. However, this study does not exclude the possibility of the presence of primitive osteogenic precursors in the STRO-1⁻ fraction of the BM.
In our study, CFU-F could not be grown from the STRO-1- fraction of BM. Previous studies have shown that the STRO-1+ population contains cells with the potential to develop into a number of distinct stromal cell types, including fibroblasts, smooth muscle cells, and adipocytes. The present work therefore extends the range of cell types into which STRO-1+ can develop. At a clonal level, approximately 50% of the CFU-F examined showed the capacity to differentiate along both the osteogenic and adipocytic cell lineages. It will be of interest to determine whether, in adult BM, all four stromal elements arise from a common multipotential stromal precursor population as previously reported by others. The answer to this question must await the development of techniques to genetically mark putative stromal stem cells and to follow their progeny.

In conclusion, this study shows that osteoprogenitors in human BM are restricted to a subpopulation of cells that express the STRO-1 antigen. These data provide an important basis from which to attempt further enrichment and characterization of osteoprogenitor cells, in particular to investigate more stringently their requirements for growth and differentiation and to identify culture conditions that lead to the expansion of their number in vitro, with a view toward potential clinical application of these cells.

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