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Plastic Adherent Stromal Cells From the Bone Marrow of Commonly Used Strains of Inbred Mice: Variations in Yield, Growth, and Differentiation

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Abstract Bone marrow stroma contains a unique cell population, referred to as marrow stromal cells (MSCs), capable of differentiating along multiple mesenchymal cell lineages. A standard liquid culture system has been developed to isolate MSCs from whole marrow by their adherence to plastic wherein the cells grow as clonal populations derived from a single precursor termed the colony-forming-unit fibroblast (CFU-F). Using this liquid culture system, we demonstrate that the relative abundance of MSCs in the bone marrow of five commonly used inbred strains of mice varies as much as 10-fold, and that the cells also exhibit markedly disparate levels of alkaline phosphatase expression, an early marker of osteoblast differentiation. For each strain examined, the method of isolating MSCs by plastic adherence yields a heterogeneous cell population. These plastic adherent cells also exhibit widely varying growth kinetics between the different strains. Importantly, of three inbred strains commonly used to prepare transgenic mice that we examined, only cells derived from FVB/N marrow readily expand in culture. Further analysis of cultures derived from FVB/N marrow showed that most plastic adherent cells express CD11b and CD45, epitopes of lymphohematopoietic cells. The later consists of both pre-B-cell progenitors, granulocytic and monocytic precursors, and macrophages. However, a subpopulation of the MSCs appear to represent bona fide mesenchymal progenitors, as cells can be induced to differentiate into osteoblasts and adipocytes after exposure to dexamethasone and into myoblasts after exposure to amphotericin B. Our results point to significant strain differences in the properties of MSCs and indicate that standard methods cannot be applied to murine bone marrow to isolate relatively pure populations of MSCs. J. Cell. Biochem. 72:570–585, 1999.

Key words: bone marrow stroma; marrow stromal cells; colony-forming unit-fibroblast

Bone marrow provides a continual source of circulating red cells, platelets, monocytes, granulocytes, and lymphocytes. Each of these cell populations is derived from a common hematopoietic stem cell (HSC). Many of the factors, both secreted and cell-associated, required for the lineage commitment of HSCs are produced by the marrow stroma. The stroma is a complex tissue, composed of a number of vascular and connective tissue cell types including endothelial cells, smooth muscle cells, adipocytes, osteogenic cells, and stromal cells. Over the past several decades, a large body of literature has documented that marrow stromal cells (MSCs), in particular, play a vital role in the subsistence, growth, and differentiation of HSCs [Muller-Sieburg and Deryugina, 1995; Oostendorp and Dormer, 1997; Dexter et al., 1984; Deryugina and Muller-Sieburg, 1993]. As a result, MSCs are chiefly regarded as hematopoietic support cells, and are used exclusively as feeder layers to culture HSCs in vitro.

During the 1970s, Friedenstein [1976] was the first to report that MSCs also posses osteogenic potential. He initially exploited the propensity of these cells to adhere to tissue culture plastic as a means to isolate them from bone marrow. Friedenstein noted that under these conditions, the MSCs grew as foci with a fibroblast-like morphology. Since the foci appeared clonal in nature, the cells were termed colony-forming unit-fibroblasts (CFU-F) after the nomenclature used in colony assays of hematopoietic precursors. The relative abundance of MSCs

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in marrow is routinely determined by counting the number of these clonogenic precursors, or CFU-Fs. Importantly, Friedenstein et al. [1987] demonstrated that MSCs derived from bone marrow but not other tissues such as spleen or thymus form a fibrous tissue and bone when incubated within diffusion chambers implanted in vivo. Subsequently, Owen and Friedenstein [1988] proposed that MSCs represent precursors of adipocytes and osteoprogenitor cells in marrow.

Many different groups have since confirmed the osteogenic potential of MSCs first described by Friedenstein. In a modification of the original diffusion chamber assay, quail [Goshima et al., 1991a], rat [Goshima et al., 1991b], and human [Kuznetsov et al., 1997] MSCs were shown to form bone and cartilage when impregnated into ceramic cubes or powder and implanted in vivo. Human MSCs loaded into ceramic carriers have also been shown to augment the repair of segmental defects in the long bones of dogs [Kadiyala et al., 1997]. More recently, several laboratories have documented that MSCs cultured in vitro can be induced to differentiate into osteoblasts [Leboy et al., 1991; Rickard et al., 1996], chondrocytes [Ashton et al., 1980], adipocytes [Lanotte et al., 1982; Bennet et al., 1991], and myoblasts [Wakitani et al., 1995]. Collectively, these results have led to the hypothesis that MSCs represent a unique cell population capable of differentiating along multiple mesenchymal cell lineages, and hence represent stem cells for nonlymphohematopoietic tissues [Owen, 1988; Caplan, 1991].

Marrow stromal cells have been isolated by their adherence to plastic from a variety of species, including mouse [Friedenstein et al., 1976; Wang and Wolf, 1990], rat [Simmons et al., 1991], guinea pig [Friedenstein, 1976], rabbit [Friedenstein, 1976; Ashton et al., 1984], pig [Thomson et al., 1993], monkey [Kramvis et al., 1984], and human subjects [Castro-Malaspina et al., 1980]. Although the cells are generally assumed to be similar, some of the data suggest species variations in the properties of the cells. In this report, we demonstrate that plastic adherent cell cultures derived from the bone marrow of five inbred strains of mice, BALB/c, C57BL/6, FVB/N, DBA/1, and 129 are composed of a heterogeneous cell population. The yield of MSCs, levels of alkaline phosphatase (ALP) expression, and the overall growth kinetics of these cultures vary dramatically between the different strains. In each case, these plastic adherent cell cultures consist predominantly of cells that express both CD11b and CD45, which include pre-B-cell progenitors, as well as granulocytic and monocytic precursors that persist in the cultures for several weeks. We estimate that MSCs represent only 10–20% of the total cell population in plastic adherent marrow cell cultures. Despite the heterogeneous nature of these cultures, MSCs can be induced to differentiate into osteoblasts and adipocytes by exposure to dexamethasone. Also, cells express a myoblast-like phenotype, fusing into contractile multinucleated fibers, after exposure to amphotericin B. Collectively, our data show that isolating MSCs by the standard method of plastic adherence does not yield a pure cell population from murine marrow, yet the small percentage of MSCs that comprise the plastic adherent cells appear to represent multipotential mesenchymal progenitors.

MATERIALS AND METHODS

Isolation and Growth of Plastic Adherent Cells from Marrow

To isolate adherent cells, femurs and tibias of 6- to 8-week-old female BALB/c, C57Bl/6, DBA/1, FVB/N, or 129 mice (Jackson Laboratories) were dissected away from attached muscle and connective tissue, the ends of the bones were removed, and marrow was extruded by inserting a 21-gauge needle into the shaft of the bone and flushing it with 1 ml of α-MEM supplemented with 10% fetal bovine serum (FBS) (Lots 6003E and 6000E; Atlanta Biologicals, Norcross, GA). The marrow plugs were dispersed by passage through a 16-gauge needle and the marrow filtered through a 75-μm filter (Falcon, Franklin Lakes, NJ). Cells were plated at a density of 20–40 $\times$ 10^6 cells per 9.5 cm^2. Unless indicated otherwise, the nonadherent cell population was removed after 72 h and the adherent layer washed once with fresh media; the cells were then continuously cultured for 1–4 weeks. Where indicated, human insulin-like growth factor-1, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), or murine epidermal growth factor (EGF) (Gibco, Grand Island, NY) was added to cultures immediately after harvest, and the cultures were typically refed every 48 h with growth factor-supplemented media. Cells were isolated by treating with 0.25% trypsin containing 50 mM EDTA for 5 min at room tempera-

ture and then gently scraping with a plastic cell scraper (Fischer Scientific, Boston, MA). Cells were typically diluted 1:3 or 1:4 at each passage. The number of CFU-Fs was determined by fixing cultures in ice-cold methanol for 5 min, staining with Geimsa for 15 min, and then counting the number of colonies of >2-mm diameter.

Assay of Alkaline Phosphatase (ALP) Activity

Plastic adherent cells were collected by centrifugation at 2,000*g* for 10 min at room temperature. The cell pellets were resuspended in 100–200 µl of lysis buffer [1.5 M Tris(hydroxymethyl)aminomethane, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 1% Triton X-100, pH 9.2] and incubated at 37°C for 30 min. The protein content of each lysate was measured using a commercial assay kit (Protein Assay; Bio-Rad, Hercules, CA) as described by the manufacturer. Aliquots of the cell extract (5–20 µl) were added to alkaline buffer (Sigma 221; Sigma Chemical Co., St. Louis, MO) containing 5 mM p-nitrophenyl phosphate (Sigma 104) and the rate of p-nitrophenol production was measured at 410 nm. The specific activity of the cell extract was determined by assuming that 1 O.D. unit at 410 nm is equivalent to 64 nmoles of p-nitrophenol.

Growth of Murine Marrow Cells on Coated Plates

Murine bone marrow was plated directly onto 6-well plates coated with various extracellular matrix proteins (Biocoat; Falcon) at a density of 25×10^6 cells per 9.5 cm^2. Cells were cultured for 8 days after which the number of CFU-Fs was determined. ALP activity and cell number were measured after 13 days of continuous culture. Cell counts were made using a hemocytometer.

Assay of Hematopoietic Progenitors

Plastic adherent cells cultured for 10 days (first passage) or passaged at a dilution of 1:3 and cultured an additional 10 days (second passage) were resuspended in methylcellulose-based media (Methocult, Stem Cell Technologies, Vancouver, BC, Canada) at various densities. To assay for the presence of pre-B-cell progenitors, cells were resuspended in methylcellulose based media supplemented with recombinant human interleukin-7 (IL-7). To assay for the presence of colony-forming unit-granulocyte (CFU-G), colony-forming unit-monocyte (CFU-M), and colony-forming unit-granulocyte/monocyte (CFU-GM), cells were resuspended in methylcellulose-based media supplemented with pokeweed mitogen-stimulated murine spleen cell-conditioned media. The semisolid cell suspension was plated in a 60-mm^2 dish and scored for the number of nonadherent colonies after 12 days in culture. Two replicate plates were made for each sample and a mean calculated.

Immunofluorescence and Histology

Nonadherent cell colonies were harvested from methylcellulose cultures by centrifugation at 1,000*g* for 15 min. Pellets were washed and resuspended in phosphate-buffered saline (PBS) (pH 7.4), counted on a hemocytometer, and adjusted to a final density of 10^5 cells/ml. Aliquots (1 ml) were transferred to cytofunnels (Shandon, Pittsburgh, PA) and centrifuged at 500*g* for 15 min. Cytospins were washed twice with PBS (pH 7.4), air dried, and fixed with cold acetone for 2 min. Cytospins derived from the pre-B-cell progenitor methylcellulose assays were hydrated with PBS/0.1% bovine serum albumin (BSA) for 15 min, incubated with rat anti-mouse CD16/CD32 (Pharmingen Fc block) at a dilution of 1:50 for 30 min, and washed in PBS/BSA. Cells were then incubated with 20% filtered normal rabbit sera for 30 min, followed by FITC-conjugated rabbit anti-mouse Ig (Dako, Glostrup, Denmark) at a dilution of 1:40 for 1 h. Slides were immersed for 5 min in PBS, washed twice in dH_2O, and coverslipped with an immunofluorescent aqueous mounting media (Dako). Plastic adherent cultures grown on chamber slides (Falcon) were similarly fixed, incubated with the Fc block, incubated with normal goat serum and a biotinylated CD11b antibody (Pharmingen, San Diego, CA) at a dilution of 1:300, or rabbit serum and a rat anti-pan endothelial cell antibody (Pharmingen) at a dilution of 1:200. Primary antibodies were detected by incubating 30 min with an FITC-conjugated ant-biotin antibody (Dako) at a dilution of 1:100 or an FITC-conjugated rabbit anti-rat antibody (Dako) at a dilution of 1:40, respectively. Slides were counterstained with either ethidium bromide or 4′-6-diamidino-2-phenylindole (DAPI).

First-passage murine plastic adherent cells grown on chamber slides were fixed as described above and stained with toluidene blue for 5 s. Slides were rinsed with 95% ethanol and air dried. Slides were covered with immersion

oil and photographed without coverslips. Cytospins derived from the CFU-G, CFU-M, and CFU-GM methylcellulose assays were stained with periodic acid-Schiff (PAS) (Sigma) as per the manufacturer's instructions. Cytochemical staining for alkaline phosphatase expression was done using a commercial kit (Sigma, #85L-2) according to the manufacturers instructions. All micrographs were photographed using a fluorescent microscope (Nikon Optiphot 2).

Differentiation of Marrow Stromal Cells

To induce an osteogenic phenotype, plastic adherent cultures were refed daily beginning on day 7 with osteo-inductive media (α-MEM supplemented with 10% fetal bovine serum (FBS), 10 mM β-glycerol phosphate, 50 μg/ml ascorbic acid, and 10^{-8} M dexamethasone) for ≤3 weeks. Mineralization of the extracellular matrix was visualized by staining with Alizarin Red S. Briefly, cell monolayers were washed with PBS, stained for 5 min with a 2% (w/v) solution of Alizaren Red S adjusted to pH 4.1 with ammonium hydroxide, and then washed with water.

To induce adipocyte differentiation, cells were cultured as described above for 10–12 days and then passaged at a dilution of 1:9. The passaged cells were treated with 5 μg/ml insulin and 10^{-9} M dexamethasone for ≤3 weeks. Myotube formation was induced by culturing first-passage cells in 1.5 μg/ml amphotericin B for approximately 2 weeks and then returning the cells to media devoid of the fungicide. Myotubes typically appeared approximately 1 week after the amphotericin B was withdrawn.

RESULTS

Properties of Plastic Adherent Cells from Different Inbred Strains of Mice

Work in our own laboratory demonstrated striking dissimilarities in the composition, growth, and differentiation potential of MSCs cultured from the bone marrow of rodents and humans (data not shown). Therefore, we investigated whether similar differences exit between MSCs isolated from different inbred strains of mice. Using standard methods of isolating MSCs from rat [Simmons et al., 1991], mice [Piersma et al., 1983], rabbit [Friedenstein, 1976], and human [Rickard et al., 1996; Castro-Malaspina et al., 1980; Kimura et al., 1988] by adherence to plastic, Figure 1 illustrates that the number of CFU-Fs derived from the bone marrow of five different inbred mouse strains varies dramatically. The bone marrow of BALB/c mice yield the highest number of CFU-Fs, with an estimated frequency of 3 ± 0.22 per 10^6 total cells. FVB/N marrow contained 2.1 ± 0.22 and DBA/1 contained 1.5 ± 0.1 CFU-Fs per 10^6 cells, which represent 30% and 50% fewer CFU-Fs as compared with

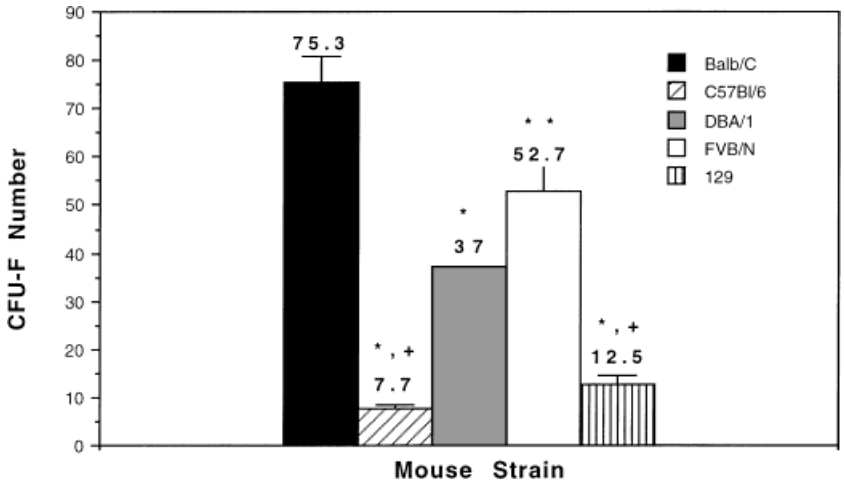

Fig. 1. Bone marrow from different inbred mouse strains contain varying numbers of CFU-Fs. Bone marrow harvested from four individuals of each indicated mouse strains were pooled and plated at a density of 25 × 10^6 cells per 9.5 cm^2 and cultured for 72 h. The nonadherent cell fraction was then removed, and the cultures incubated an additional 5 days. Cultures were then fixed and stained and the number of CFU-Fs counted. For each sample a mean value was reported from three replicate plates; standard deviation of the data points is illustrated by bars. The number of CFU-Fs in Balb/C marrow was significantly different from that of other strains (**, $P \leq 0.01$; *, $P \leq 0.005$; Student's t-test). The number of CFU-Fs in FVB/N marrow was also significantly different than that of C57Bl/6 and 129 strains (+, $P \leq 0.005$; Student's t-test).

BALB/c bone marrow, respectively. Remarkably, the number of CFU-Fs in the bone marrow from C57Bl/6 (0.3 ± 0.22) and 129 (0.5 ± 0.08) strains is approximately 10-fold lower than from BALB/c mice.

Plastic adherent cell cultures established from the marrow of each mouse strain consisted of a heterogeneous mixture of morphologically distinct cell types. The growth kinetics of these cultures also vary widely between strains (Fig. 2A). Plastic adherent cell cultures established from BALB/c bone marrow proliferate at a rate of 31,300 ± 2,828 cells per day based on the slope of the proliferation curved calculated from day 8 to 18. This rate is approximately 1.5- and 4-fold greater than that calculated for plastic adherent cultures derived from FVB/N (19,800 ± 2828) or DBA/1 (7,044 ± 5,088) bone marrow, respectively. By contrast, the growth rate of plastic adherent cultures from C57Bl/6 (−628 ± 114) and 129 (1,638 ± 486) marrow explants were so low that the cultures fail to thrive in vitro. The total number of cells after 18 days in culture from Balb/C and FVB/N marrow explants was significantly greater than that of C57Bl/6, DBA/1, and 129 strains ($p \leq 0.05$, Student's t-test), and growth rates were also significantly greater than C57Bl/6 and 129 cultures ($p \leq 0.05$, Student's t-test).

Levels of ALP expression, an early marker in the program of osteoblast differentiation, within these cultures also differed between strains. Cultures derived from BALB/c, C57Bl/6, and 129 mice express low levels of ALP over a period

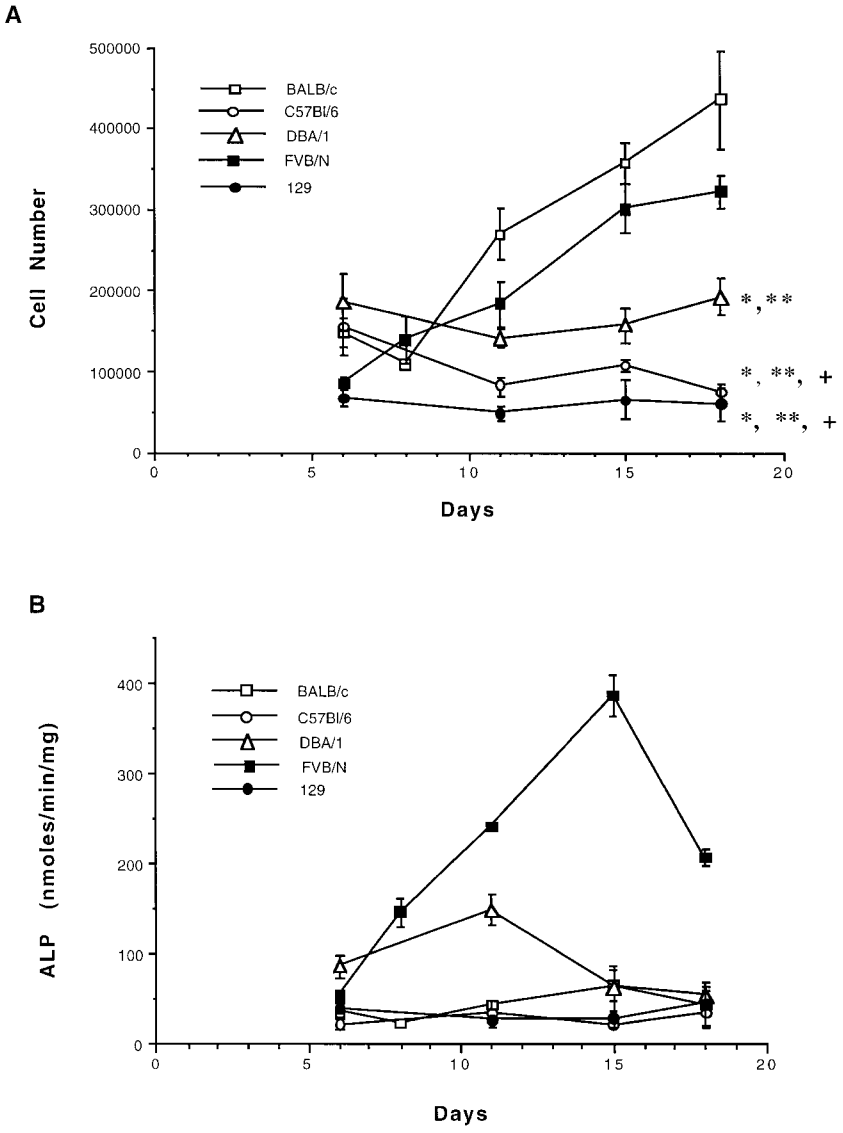

Fig. 2. Growth kinetics and ALP activity of plastic adherent cells derived from the bone marrow of different inbred mouse strains. Bone marrow harvested from the indicated mouse strains was plated at a density of 25×10^6 cells per 9.5 cm^2 and cultured for 72 h, after which the nonadherent cell fraction was removed. Cultures were continually incubated and at the indicated time points the total cell number **(A)** and alkaline phosphatase activity **(B)** were measured. Data are expressed as a mean of two replica plates per time point; bars represent the standard deviation of the data points. The slope of each proliferation curve was calculated using data points from 8 to 18 days. The total cell number at day 18 for Balb/C and FVB/N cultures was significantly different from C57Bl/6, DBA/1, and 129 strains (*, $P \leq 0.05$; Student's t-test). The growth rate of Balb/C cultures was significantly different from C57Bl/6, DBA/1, and 129 strains (**, $P \leq 0.05$; Student's t-test), and that of FVB/N cultures also significantly different from C57Bl/6 and 129 strains (+, $P \leq 0.05$; Student's t-test).

of 3 weeks (Fig. 2B). By contrast, ALP activity in FVB/N derived cultures steadily increases for the first two weeks, peaking at a value approximately 6-fold greater as compared with the BALB/c culture. In DBA/1 cultures, ALP expression peaks after 11 days, reaching a level approximately 3.5-fold greater than BALB/c cultures. Cytochemical staining of these cultures indicate that in each case, only the fibroblastoid-shaped MSCs express appreciable levels of ALP. These data indicate that large disparities exist in the yield and pattern of ALP expression in MSCs derived from different inbred strains of mice. Because marrow donors from each strain were 6- to 8-week-old females, differences in age and gender likely do not account for these differences. Moreover, as there is little variability in the average weight of mice from the different strains, significant differences in bone mass and volume cannot account for the variation in MSC abundance. Importantly, of the three strains commonly used to prepare transgenics; C57Bl/6, 129, and FVB/N, only the latter provides a ready source of MSCs using standard isolation procedures.

Isolation of Stromal Cells from FVB/N Bone Marrow

As stated above, microscopic examination revealed that plastic adherent cell cultures derived from each mouse strain consisted of a heterogeneous collection of morphologically distinctive cell types (data not shown). To better characterize the nature of these plastic adherent cells, we examined cultures derived from FVB/N bone marrow in greater detail. The FVB/N strain was chosen because (1) primary cultures exhibited good growth rates; (2) the cells showed a propensity to upregulate ALP expression, indicating they potentially can differentiate along the osteoblast lineage; (3) the strain is one of three strains commonly used to prepare transgenic mice; and (4) our laboratory has previously developed transgenic mouse models of osteogenesis imperfecta in an FVB/N background [Khillan et al., 1991], providing a model system to evaluate the therapeutic value of bone marrow stromal cells in treating bone disease.

Initially, we sought the most effective plating strategy to isolate MSCs from whole marrow by their attachment to plastic. Therefore, we measured CFU-F numbers as a function of the time whole marrow is initially cultured before removal of the nonadherent cell population. As shown in Figure 3, when whole bone marrow was cultured for 4, 24, or 72 h, the yield of CFU-Fs increased linearly as a function of time. However, when whole marrow was cultured for 144 h, there was no significant increase in the yield of CFU-Fs as compared with 72 h (data not shown). Therefore, our standard practice of culturing whole marrow for 72 h prior to removal of the nonadherent cell population yields the greatest number of CFU-Fs. Also, these data show that only a small number of CFU-Fs are recovered from each nonadherent cell fraction upon replating.

Similarly, we compared the yield of CFU-Fs provided by different plastic-coated surfaces.

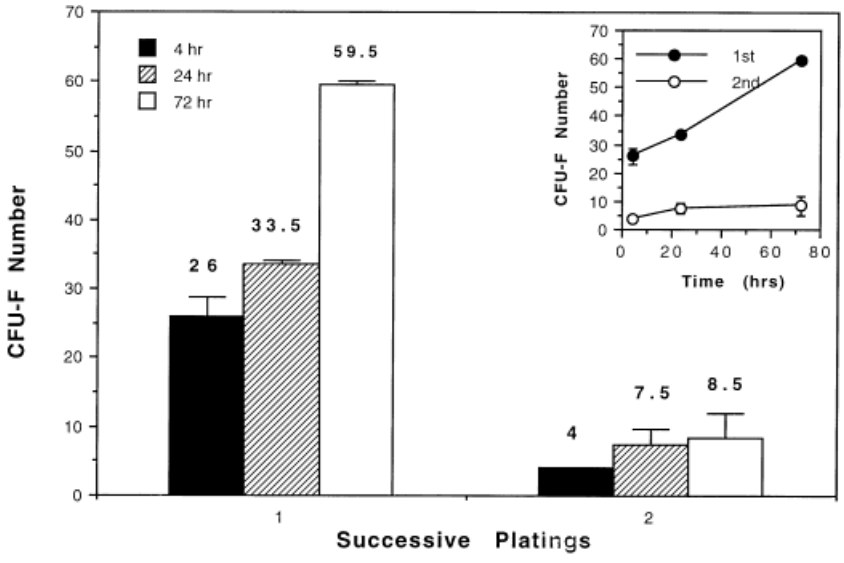

Fig. 3. Optimization of stromal cell yields from whole bone marrow. Whole bone marrow from FVB/N mice was plated in plastic dishes, and at the times indicated, the nonadherent cell population was removed and the adherent cells washed and refed with fresh media. The nonadherent fraction was replated into a fresh tissue culture plate and the process repeated. The adherent cells were cultured a total of 9 days, after which the number of CFU-Fs were counted. The data are expressed as a mean of two replicate plates per time point; bars represent the standard deviation of the data points. Inset, same data points plotted as a line graph.

As shown in Figure 4A, untreated plastic plates yield the highest number of CFU-Fs (88.3 ± 6.8 per 25×10^6 nucleated cells plated). This number was significantly greater than the yield of CFU-Fs on collagen type I and collagen type IV coated plates ($p \leq 0.01$) or on laminin-coated plates ($p \leq 0.005$). By contrast, when the total cell number on each plate was counted, the only surface that yielded significantly fewer cells as compared with untreated plastic was laminin-coated plates ($p \leq 0.01$) (Fig. 4B). Lastly, cultures grown on different coated surfaces exhibited similar levels of ALP expression, with the exception of Poly-D-lysine. Cells cultured on the latter surface expressed 16-fold higher ALP level as compared with cells plated on plastic (Fig. 4C). Hence, poly-D-lysine appears to stimulate the differentiation of cells toward an osteoblast phenotype. Collectively, these results indicate that standard tissue culture plastic ware is best suited for the isolation and growth of MSCs.

Growth Kinetics of FVB/N Plastic Adherent Cells

Immediately after plating, plastic adherent cells from FVB/N marrow remain relatively quiescent during the first 3–4 days in culture. After this time the cells enter a proliferative phase with growth kinetics as depicted in Figure 5A. Over the same time period ALP activity initially rises slowly, peaks at a high level, and

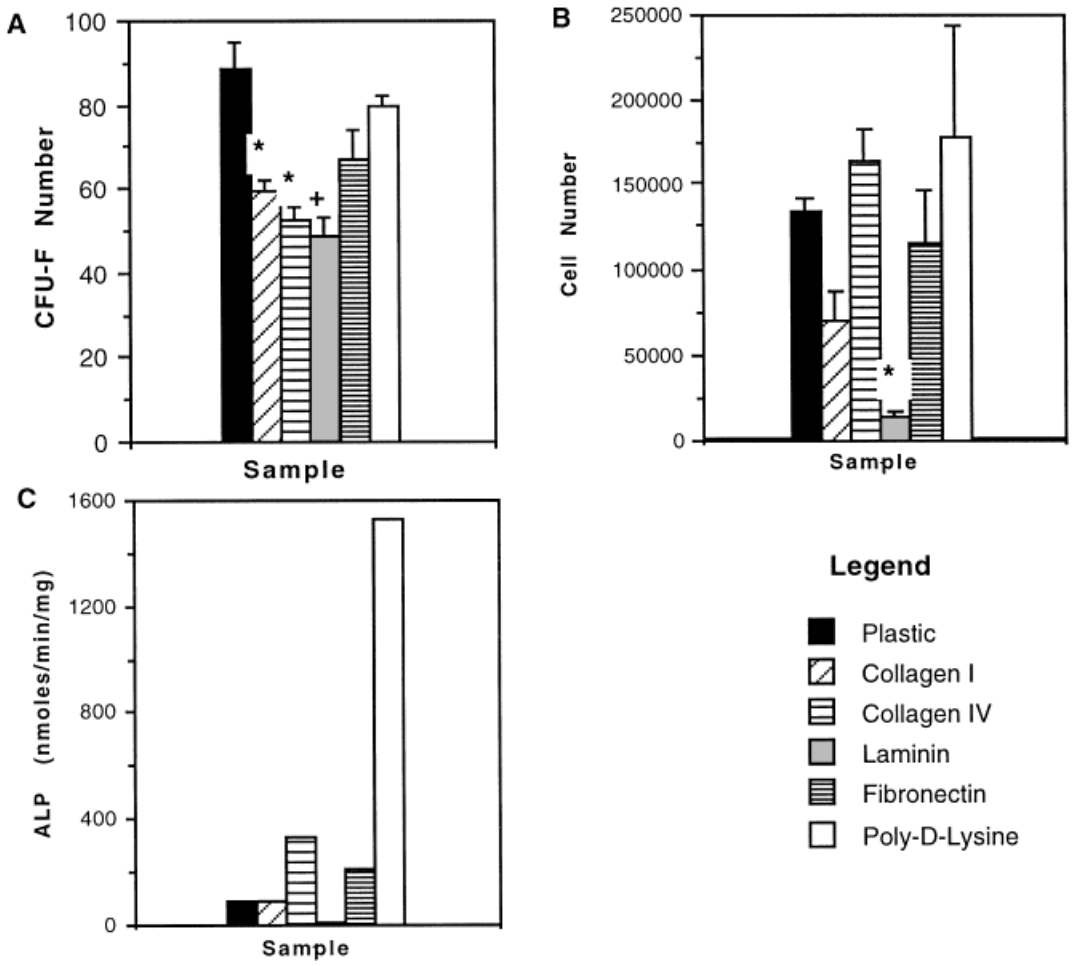

Fig. 4. Effect of different plating surfaces on growth and ALP expression of plastic adherent cells. FVB/N bone marrow was cultured on plastic dishes or plates coated with the indicated extracellular matrix proteins for a total of 8 days and the number of CFU-Fs **(A)** was determined. After 13 days of continuous culture the total number of cells **(B)** and levels of ALP expression **(C)** were also determined. The number of CFU-Fs and total cell number measurement were done in triplicate and plotted as a mean value. The error bars represent the standard deviation of the data points. The number of CFU-Fs in plastic plates was significantly greater than in collagen type I and collagen type IV coated plates (*, $P \leq 0.01$; Student's t-test) and laminin coated plates (+, $P \leq 0.005$; Student's t-test). Also, the total number of cells on laminin coated plates was significantly less as compared with plastic.

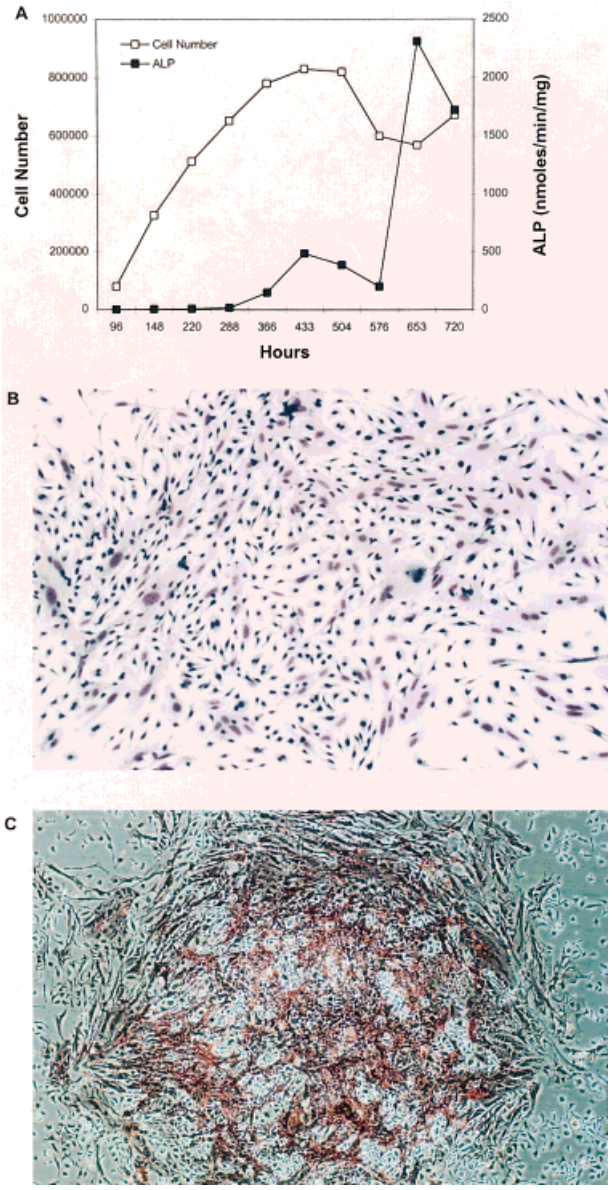

Fig. 5. Growth kinetics and ALP activity of FVB/N plastic adherent cells.. **A:** Plastic adherent cells from FVB/N mice were continuously cultured for 4 weeks, over which time both cell number and ALP activity was measured. **B:** Photomicrograph of a typical foci from 11-day FVB/N plastic adherent cultures stained with Giemsa. Note the variety of morphologically distinct cells interspersed between the fibroblastoid-shaped stromal cells. **C:** Photomicrograph of a typical foci from a 3-week-old FVB/N plastic adherent culture after staining for expression of ALP activity (red). ×100.

finally declines. The cultures contain fibroblastoid-shaped MSCs that grow as foci, together with round and stellate cells found within the foci and dispersed between them (Fig. 5B). As shown in Figure 5C, fibroblastoid-shaped MSCs within the foci have high levels of ALP expression.

Heterogeneity of FVB/N Plastic Adherent Cultures

Most round and stellate-shaped cells in FVB/N plastic adherent cultures stain positive for expression of CD11b (Fig. 6A). The CD45 receptor displayed a similar pattern of expression (data not shown). Therefore, the cultures contain lymphohematopoietic cells, which we estimate constitute approximately 80% of the adherent cell population. The CD11b and CD45 positive cells persist in these cultures after several weeks, even after the cells are passaged (Fig. 6B). Several clusters of endothelial cells are also evident within these cultures, as illustrated by staining with a pan-endothelial marker (Fig. 6C). MSCs that exhibit a fibroblastoid morphology do not express the aforementioned antigens, but stain for expression of collagen type I and vimentin (data not shown). Immunostaining of adherent cell cultures from

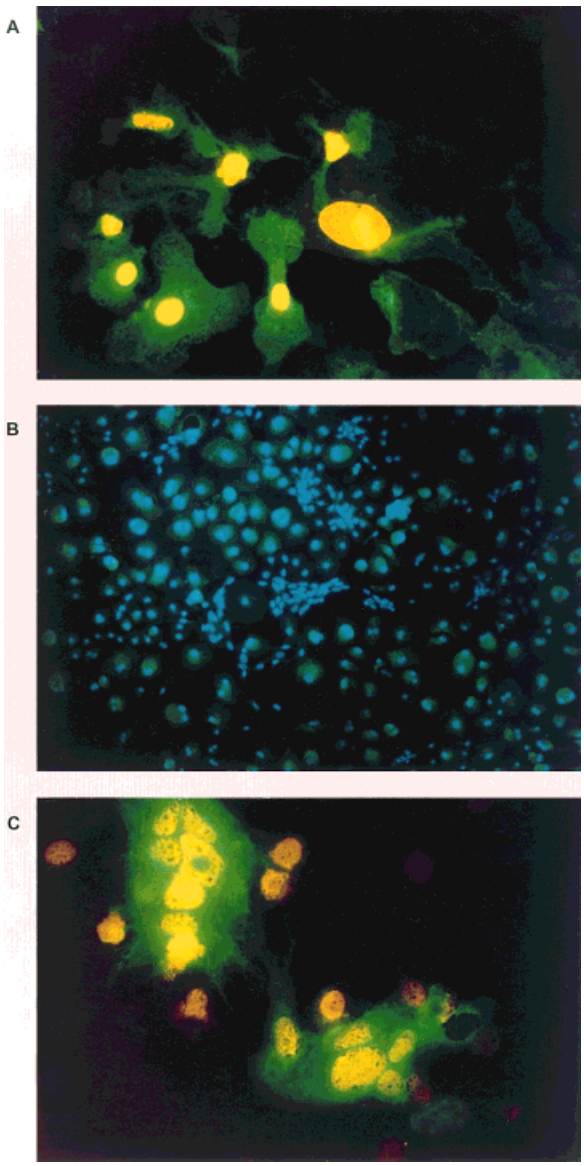

Fig. 6. Characterization of plastic adherent cultures by immunofluorescence. **A:** Plastic adherent cells cultured for 11 days were fixed and stained with an anti-CD11b antibody. Nuclei were counter stained with ethidium bromide. ×400. **B:** Plastic adherent cells cultured for 9 days were passaged 1:2 and cultured an additional 5 days before staining with an anti-CD11b antibody. Nuclei were counterstained with DAPI. ×100. **C:** Cells cultured as in (A) were stained with an anti-pan-endothelial antibody and counterstained with ethidium bromide. ×400.

BALB/c and DBA/1 marrow showed similar proportions of contaminating lymphohematopoietic cells.

Both first- and second-passage plastic adherent cells yielded pre-B-cell colonies when plated in methylcellulose based media (Fig. 7A). The number of colonies was proportional to the number of plastic adherent cells plated for both first-passage ($r^2 = 0.98$) and second-passage ($r^2 = 0.74$) cultures. Immunofluorescent staining with an anti-murine immunoglobulin antibody confirmed that colony-derived cells were of the B-cell lineage (Fig. 7C). Similarly, the number of granulocyte and/or monocyte progenitors in plastic adherent cultures was proportional to the number of first-passage ($r^2 = 0.95$) or second-passage ($r^2 = 0.96$) cells plated (Fig. 7B). In this case, colony-derived cells stained positive for expression of CD11b (data not shown) and PAS (Fig. 7D), indicating the presence of myeloid lineage cells. Both first- and second-passage cells plated in media containing only 0.9% methylcellulose (no added cytokines) failed to produce any lymphohematopoietic colonies..

Differentiation Potential of FVB/N Plastic Adherent Cells

Plastic adherent FVB/N cultures also contain cells capable of differentiating into osteoblasts, adipocytes, and myoblasts. Plastic adherent cells that were cultured 7 days, and then treated for 2 weeks with osteo-inductive media, expressed high ALP activity and deposited calcium in the extracellular matrix as evidenced by staining with Alizaren Red S (Fig. 8A). Using reverse transcription-polymerase chain reaction (RT-PCR), we confirmed that expression of osteopontin, osteocalcin, and bone sialoprotein were induced to high levels after exposure of cells to osteo-inductive media. By contrast, levels of parathyroid hormone receptor expression, which is specifically induced in response to vitamin D, remained unchanged (data not shown). Hence, cells exposed to osteo-inductive media express a number of bone specific genes. In addition, exposure of first-passage cells to dexamethasone for several weeks induces the accumulation of lipid droplets in a large percentage of cells (Fig. 8B). Similarly, exposure of MSCs to the peroxisome proliferator-activated receptor γ2 (PPARγ2) agonist 5,8,11,14-eicosatetraynoic acid (EYTA) rapidly induces the accumulation of multi-ocular lipoblasts similar to that seen after prolonged exposure to dexamethasone (data not shown). Because PPARγ2 is expressed specifically in adipocytes [Tontonoz et al., 1994), the ability of MSCs to respond to EYTA provides further evidence of

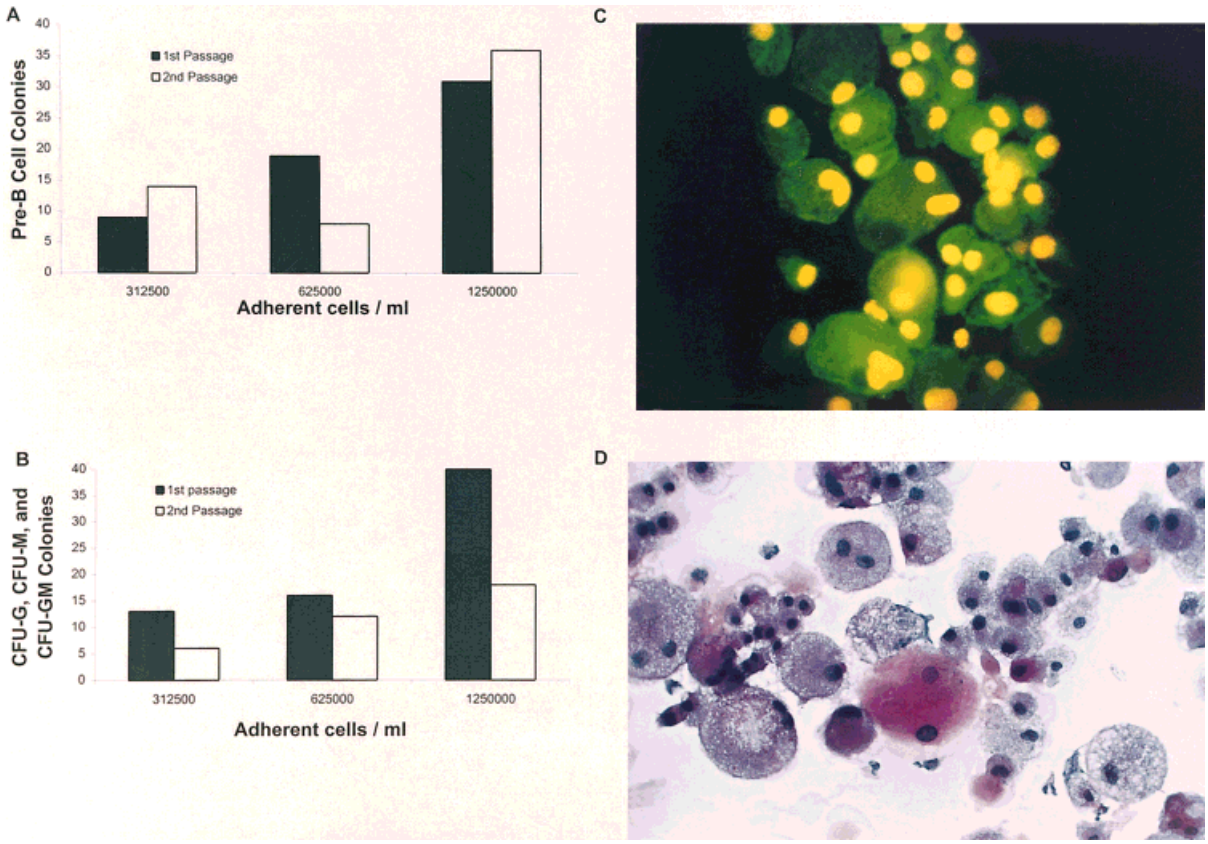

Fig. 7. Colony-forming assays for granulocyte, monocyte, and pre-B-cell progenitors. Plastic adherent cells from FVB/N marrow cultured for 10 days (first passage) or passaged at a dilution of 1:3 and cultured an additional 10 days (second passage) were resuspended at the indicated cell density in Methocult media to assay for the existence of **(A)** pre-B-cell progenitors or **(B)** colony-forming units of granulocytes (CFU-G), monocytes (CFU-M), and granulocytes and monocytes (CFU-GM) respectively. For each sample, replicates were made and the number of nonadherent colonies determined. **C:** Photomicrograph of pre-B-cell colony-derived cells stained with a murine anti-immunoglobulin antibody and counter stained with ethidium bromide. **D:** Photomicrograph of CFU-G, CFU-M, CFU-GM colony-derived cells stained with PAS and counterstained with Gill's hematoxylin. ×400.

their capacity to differentiate into adipocytes. Finally, exposure of cells to amphotericin B produced a network of multinucleated fibers (Fig. 8C). The fibers rhythmically contract when exposed to heat and hence appear to represent myotubes.

Effect of Polypeptide Growth Factors on the Growth and Differentiation of FVB/N Plastic Adherent Cells

Continuous culture of FVB/N plastic adherent cells in media supplemented with 2.5 ng/ml bFGF and 10 ng/ml IGF-I dramatically inhibits ALP induction in MSCs for ≤34 days in culture (Fig. 9A). Importantly, after removal of the growth factors, MSCs still respond to osteoinductive media by demonstrating a dramatic increase in ALP expression. Hence, growth factor treatment does not alter the differentiation potential of the cells. These factors had a modest effect on cell growth (data not shown).

As shown in Figure 10A, continuous exposure of plastic adherent cultures to 5 ng/ml PDGF-ββ increases the total cell number by 2-fold. This effect is not evident at a concentration of 1 ng/ml, but 10 ng/ml slightly stimulates growth, as well. Similarly, EGF at a concentration of 5 ng/ml also increases the total cell number by approximately 2-fold (Fig. 10C). By contrast, 2.5–20 ng/ml of bFGF had no effect on cell growth (Fig. 10B). Additionally, PDGF-ββ markedly reduces the level of ALP, with 10 ng/ml PDGF-ββ depressing ALP expression approximately 6-fold as compared with the control (Fig. 10D). Expression of ALP was reduced 25-fold as compared with untreated control cultures by treatment with 2.5 ng/ml bFGF (Fig. 10E). By contrast, EGF appears to in-

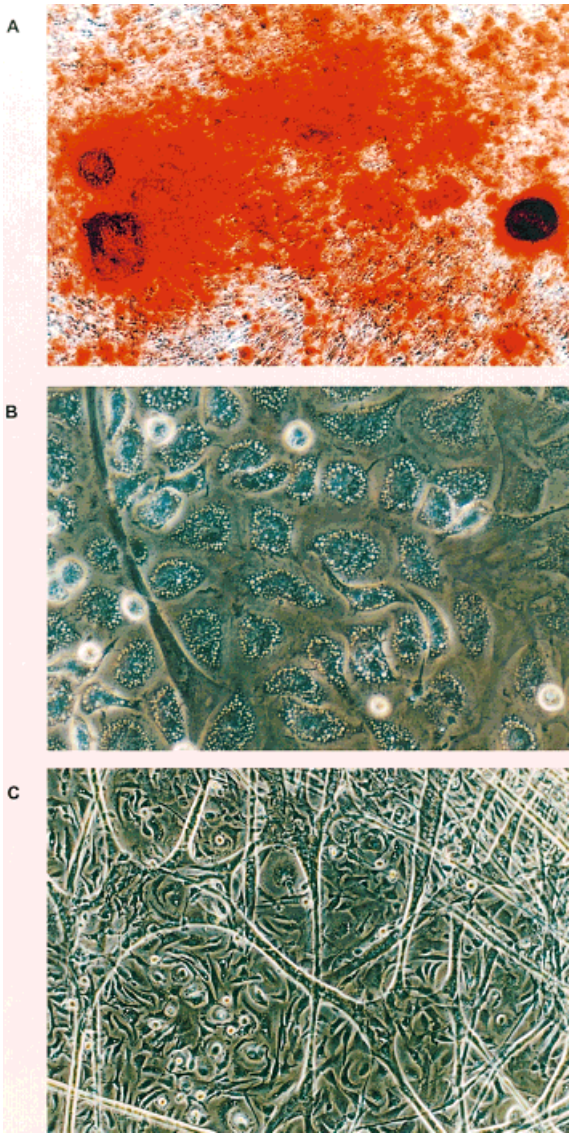

Fig. 8. Differentiation of plastic adherent cells into osteoblasts, adipocytes, and myoblasts. **A:** Phase contrast micrograph of plastic adherent cells cultured for approximately 3 weeks in osteo-inductive media and stained with Alizaren Red S. ×100. **B:** Phase contrast micrograph of plastic adherent cells continuously cultured in 1 x10^{-8}M dexamethasone for approximately 2 weeks. Note that a large number of cells contain lipid droplets. ×400. **C:** Phase contrast micrograph of myotubes generated from plastic adherent cells after exposure to amphotericin B. ×400.

crease ALP expression as compared with the control (Fig. 10F).

DISCUSSION

Friedenstein et al. [1976] originally reported that the frequency of CFU-Fs in the bone marrow of CBA × C57Bl F1 adult mice ranges from 3 to 45 per 10^6 bone marrow cells. Several other strains of mice have been reported to have similar frequencies of CFU-Fs in marrow [Xu et al., 1983]. By contrast to these reports, our data provide a direct comparison of the frequency of CFU-Fs in the bone marrow of five commonly used inbred strains of mice. We estimate the frequency of MSCs per 10^6 marrow cells as 0.3 ± 0..02 for C57Bl/6 mice, 0.5 ± 0.08 for 129 mice, 1.5 ± 0.1 for DBA/1 mice, 2.1 ± 0.22 for FVB/N mice, and 3 ± 0.22 for BALB/c mice. The number of CFU-Fs in Balb/C and FVB/N marrow are significantly greater than in C57Bl/6 and 129 strains ($p \leq 0.005$). The number of CFU-Fs in Balb/C marrow is also significantly greater than in the DBA/1 strain ($p \leq 0.01$).

For each strain examined, isolation of MSCs by plastic adherence yielded a heterogeneous cell population. The growth kinetics of the plastic adherent cells derived from different mouse strains is also highly variable. Cells derived from BALB/c and FVB/N marrow readily expand in vitro. However, the yield of plastic adherent cells is so low from C57BL/6 and 129 mice that the cultures fail to thrive in vitro.

The heterogeneous nature of adherent bone marrow cultures from mice has been reported by others. Friedenstein et al. [1976] demonstrated that marrow explants from mice contain both CFU-Fs, as well as a large percentage of histiocytes and mononuclear cells that persist in the culture for several weeks. Xu et al. [1983] demonstrated that adherent cells from $B6D2F_1$ bone marrow contain fibroblasts, macrophages, and endothelial cells. Similar results were obtained by Wang et al. [1990] in C57Bl/6 × DBA/2 F1 cultures. In the latter case, limiting dilution verified that the macrophages and endothelial cells are not derived from fibroblasts but are merely contaminants.

We extended these observations by demonstrating that plastic adherent cultures from all five mouse strains examined contain a large percentage of CD11b and CD45-positive staining cells. The CD11b antigen is expressed by monocytes, granulocytes, and natural killer (NK) cells, whereas CD45 is expressed in all lymphohematopoietic cell lineages. We estimate that 80% of cells in a primary culture stain for expression of these antigens. Importantly, colony-forming assays performed using FVB/N-derived plastic adherent cells verified the existence of pre-B-cell progenitors together with granulocytic and/or monocytic precursors

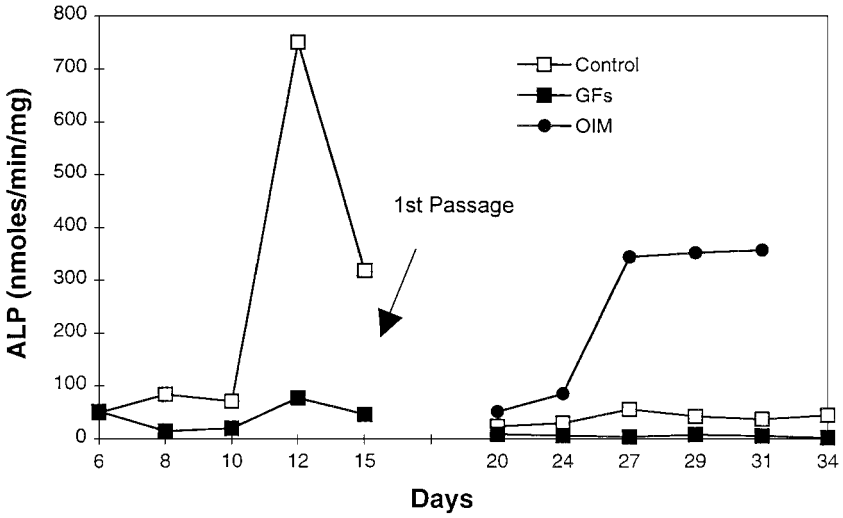

Fig. 9. Effect of basic fibroblast growth factor (bFGF) and Insulin-like growth factor-1 on growth and differentiation of plastic adherent cells. Plastic adherent cultures were established from FVB/N bone marrow as described under Materials and Methods. Cultures were either untreated (control, □) or treated with 2.5 ng/ml bFGF and 10 ng/ml IGF-1 (Gfs, ■). After 15 days of continuous culture, untreated cells were harvested, passaged at a dilution of 1:3, and cultured an additional 19 days. Treated cultures were passaged 1:3 and cultured for 19 days in the continued presence of bFGF and IGF-1, or growth factors were removed and cells treated with osteo-inductive media (OIM, ●).

within these cultures. Granulocytic precursors are most evident, as demonstrated by their propensity to migrate under the cytoplasm of MSCs. This association is necessary for their maturation [Dexter et al., 1984]. These myeloid and lymphoid progenitors persist in FVB/N plastic adherent cultures over a period of 20 days, during which time the cells were replated. Kerk et al. [1985] demonstrated that murine marrow cells that adhere to plastic within 24 h are enriched for myeloid and erythroid progenitors, which is consistent with our findings. In addition, Bearpark and Gordon [1989] showed that murine marrow cells that adhere to plastic within 2 h are enriched for long-term repopulating cells capable of rescuing lethally irradiate mice. Collectively, these results indicate that murine plastic adherent cell cultures contain a variety of lymphohematopoietic progenitors. Hence, any conclusion about engraftment of murine MSCs after transplantation of this mixed cell population in vivo must be verified by unambiguous identification of the engrafted cell phenotype.

Plastic adherent cultures derived from different mouse strains also exhibit disparate levels of ALP expression, an enzyme induced during the early phases of osteoblast differentiation. This was most evident in comparing BALB/c and FVB/N derived cultures. Although cultures from these two strains proliferate with similar kinetics, FVB/N cells express 6-fold higher ALP levels than BALB/c cells over a three week culture period. Since MSCs in the adherent cultures stain strongly for ALP expression, these data suggest that FVB/N cultures contain a subpopulation of mesenchymal progenitors that progress to a different stage along the osteoblast lineage in vitro than cells in BALB/c cultures.

To prevent induction of ALP, and hence differentiation of FVB/N-derived MSCs, we cultured cells in the presence of different polypeptide growth factors. The combination of bFGF and IGF-1, factors abundant in bone matrix thought to stimulate osteoprogenitor proliferation [Delany et al., 1994; Mundy et al., 1995] slightly stimulated cell growth and dramatically suppressed ALP levels. Alone, bFGF showed no effect on cell growth but still inhibited ALP expression. These results differ from those reported by Wang et al. [1990], who demonstrated that bFGF stimulates the proliferation of CFU-Fs from C57Bl/6 × DBA/2 adult mice. These differences may result from the fact that we cannot determine how the mitogens we tested specifically affect the growth of MSCs, as our plastic adherent cultures are composed of a heterogeneous collection of cell types. However, it is also possible that strain difference may also affect the response of cells to growth factors. Our studies also revealed that PDGF-ββ inhibited ALP expression, but to a lesser extent than bFGF. However, both PDGF-ββ and EGF stimulated cell growth. These results imply that specific combinations of growth factors can be used to stimulate the growth of plastic adherent cells and also specifically depress the differentiation of FVB/N MSCs.

We demonstrated that MSCs within FVB/N plastic adherent cell cultures differentiate into

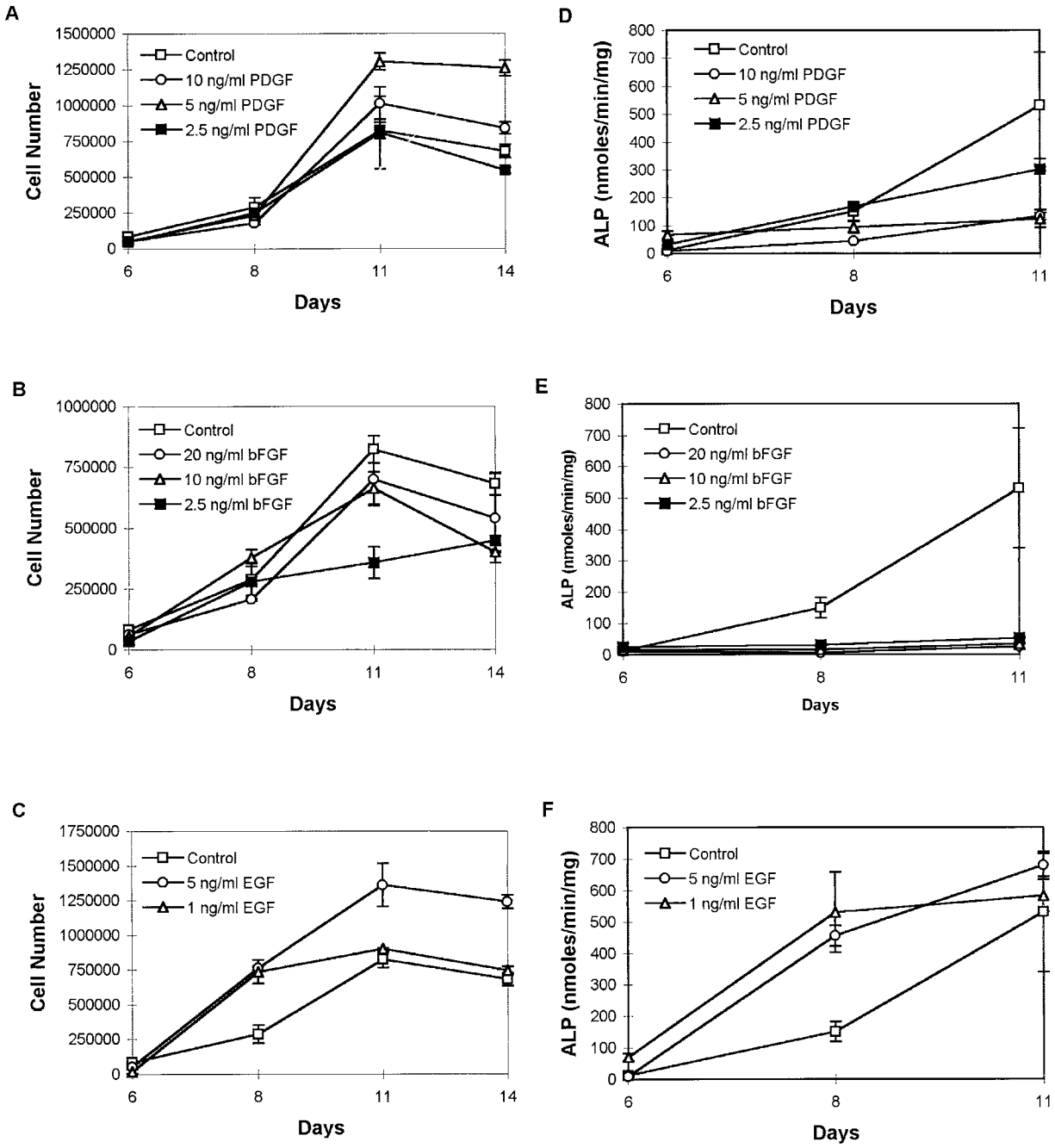

Fig. 10. Effect of platelet-derived growth factor (PDGF-ββ), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) on growth and alkaline phosphatase (ALP) expression of plastic adherent cultures. Plastic adherent cells were continuously cultured in media supplemented with the indicated concentrations of growth factors. Both cell number and ALP activity were determined at the indicated time points. Data are presented as a mean of two replica plates per time point; bars represent the range.

osteoblasts by showing that the cells express high ALP levels upon exposure to osteo-inductive media. This treatment also induces expression of several bone specific genes, nodule formation, and the deposition of a mineralized matrix. Additionally, exposure of cells to dexamethasone or the PPARγ2 agonist EYTA induced the accumulation of lipid droplets within MSCs, implying their differentiation into adipocytes. Exposure of cells to amphotericin B resulted in

the formation of multinucleated fibers that resemble myotubes, as well. Surprisingly, differentiation toward this muscle cell-like phenotype was not induced by 5-azacytidine, a reagent known to promote myoblast differentiation of embryonic fibroblasts and 10T1/2 cells [Taylor and Jones, 1982]. This drug was also recently shown to induce myoblast differentiation of rat stromal cells [Wakitani et al., 1995]. However, in these experiments cells were continuously cultured in media supplemented with amphotericin B. Hence, we believe the later agent is responsible for conversion to a muscle cell phenotype.

Both FVB/N and C57Bl/6 strains are frequently used to prepare transgenic mice because they produce a large number of high-quality embryos and superovulate in response to hormone injections [Fox and Witham, 1997]. Also, most targeted mutations are produced in embryonic stem cells derived from the 129 strain. MSCs from C57Bl/6 and 129 strains exist at a low frequency in marrow and exhibit poor growth in vitro. These properties limit the potential of these strains as sources of genetically tagged MSCs for use in homing experiments or in the generation of knockouts to study how genetic changes affect stromal cell function. Our data suggest that the FVB/N strain is best suited for this type of analysis.

It is difficult to assess why such profound differences in the properties of MSCs exit between different inbred strains of mice. In all cases, the plastic adherent cell cultures consisted of a heterogeneous collection of morphologically distinct cell types, most of which expressed CD11b. Hence, one strain did not appear to yield a more pure preparation of MSCs. There is no apparent correlation with longevity in that, for example, C57Bl/6 mice live as long as, or longer than, BALB/c mice [Fox and Witham, 1997]. Also, they do not possess any obvious pathology with their hematopoietic organs or other tissues. Apparently, differences in the properties of stromal cells merely reflects the unique genetic background of each inbred strain. One possibility is that genetic differences determine the degree to which MSCs adhere to plastic. Alternatively, genetic differences may determine the anatomical distribution of nonhematopoietic precursors so that the marrow from long bones of some mouse strains contain more cells than others.

Beamer et al. [1996] reported a profound genetic variability in the cortical thickness, density, and mineral content of bones from different inbred strains of mice. These differences were evident by 2 months of age, and persisted in 12-month-old animals. Interestingly, these parameters were highest for C3H/He mice, lowest for C57Bl/6 mice, and intermediate for BALB/c and DBA/2 mice. Comparing these results with our data on MSCs abundance in marrow from BALB/c, DBA/1, and C57BL/6 mice indicates that there may be a correlation between the cortical thickness, mineral content, and density of bone and the number of MSCs found in marrow. Since MSCs can differentiate into osteoblasts and chondrocytes, variations in MSC abundance may, in fact, directly contribute to the genetic variability in the physical properties of bone seen between inbred strains. This hypothesis is further supported by recent data from Jilka et al. [1996] who showed that a decrease in osteoblastogenesis in marrow is correlated with low bone mineral density.

Collectively, our findings raise several important points with regard to MSCs. First, it may be technically difficult to culture these cells from a particular inbred mouse strain due to their low abundance. Clearly, optimization of culture conditions may improve the growth rate of adherent cells from some mouse strains. However, the low initial yield of plastic adherent cells from the C57BL/6 and 129 strains makes it extremely difficult to test different culture conditions. Second, the ability to culture cells may also be affected by variations in the differentiation potential of MSCs. For example, FVB/N plastic adherent cells express high levels of ALP after several weeks in culture. The cells also deposit large amounts of extracellular matrix. As a result, the cells are difficult to lift from their substratum and can only be expanded for two or three passages. Third, differences in differentiation potential in vitro may also be manifested in vivo. The later may explain differing results obtained from individual labs that utilize similar experimental designs but employ MSCs from different strains of mice. Apparently, the selection of a particular

mouse strain will have important implications regarding the outcome of experiments using MSCs.

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