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Growth Kinetics, Self-Renewal, and the Osteogenic Potential of Purified Human Mesenchymal Stem Cells During Extensive Subcultivation and Following Cryopreservation

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Abstract Recent studies have demonstrated the existence of a subset of cells in human bone marrow capable of differentiating along multiple mesenchymal lineages. Not only do these mesenchymal stem cells (MSCs) possess multilineage developmental potential, but they may be cultured ex vivo for many passages without overt expression of a differentiated phenotype. The goals of the current study were to determine the growth kinetics, self-renewing capacity, and the osteogenic potential of purified MSCs during extensive subcultivation and following cryopreservation. Primary cultures of MSCs were established from normal iliac crest bone marrow aspirates, an aliquot was cryopreserved and thawed, and then both frozen and unfrozen populations were subcultivated in parallel for as many as 15 passages. Cells derived from each passage were assayed for their kinetics of growth and their osteogenic potential in response to an osteoinductive medium containing dexamethasone. Spindle-shaped human MSCs in primary culture exhibit a lag phase of growth, followed by a log phase, finally resulting in a growth plateau state. Passaged cultures proceed through the same stages, however, the rate of growth in log phase and the final number of cells after a fixed period in culture diminishes as a function of continued passaging. The average number of population doublings for marrow-derived adult human MSCs was determined to be 38 ± 4, at which time the cells finally became very broad and flattened before degenerating. The osteogenic potential of cells was conserved throughout every passage as evidenced by the significant increase in APase activity and formation of mineralized nodular aggregates. Furthermore, the process of cryopreserving and thawing the cells had no effect on either their growth or osteogenic differentiation. Importantly, these studies demonstrate that replicative senescence of MSCs is not a state of terminal differentiation since these cells remain capable of progressing through the osteogenic lineage. The use of population doubling potential as a measure of biological age suggests that MSCs are intermediately between embryonic and adult tissues, and as such, may provide an in situ source for mesenchymal progenitor cells throughout an adult's lifetime. J. Cell. Biochem. 64:278–294. © 1997 Wiley-Liss, Inc.

Key words: osteoblast; differentiation; replication; osteoprogenitor; bone marrow

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INTRODUCTION

Use of the term *stem* cell has generally been reserved for those cells possessing the ability to self-replicate and give rise to daughter cells which undergo an irreversible, terminal differentiation process [Hall and Watt, 1989]. Stem cells in adults have been studied extensively from the epidermis, gastrointestinal epithelium, and the hematopoietic compartment of bone marrow. Hematopoietic stem cells are perhaps the best characterized [Lemischka et al., 1986; Sachs, 1987; Spangrude et al., 1988], and are noted for their ability to give rise to multiple cellular phenotypes through lineage progression of daughter progenitor cells. Also present in adult bone marrow is a population of mesenchymal stem cells (MSCs) which give rise to multiple mesodermal tissue types, including bone and cartilage [Owen, 1985, 1988; Beresford, 1989; Caplan, 1991], tendon [Caplan et al., 1993], muscle [Wakitani et al., 1995; Saito et al., 1995], fat [Dennis and Caplan, 1996], and a marrow stromal connective tissue which sup-

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ports the differentiation of hematopoietic stem cells [Dexter and Testa, 1976; Majumdar et al., 1995].

Recently, techniques for the isolation and extensive subcultivation of human marrow-derived MSCs have been developed, along with a series of monoclonal antibody probes which react with the surface of these cells both in vitro and in situ [Haynesworth et al., 1992a,b; Bruder et al., 1995a]. These purified, culture-expanded human MSCs are capable of differentiating along the osteogenic [Haynesworth et al., 1992b; Bruder et al., 1995b], chondrogenic [Lennon et al., 1996; Johnstone et al., 1996], adipogenic [Pittenger et al., 1996], and marrow stromal lineages [Majumdar et al., 1995]. Further characterization of these cells documents their cell surface and extracellular matrix molecules [Haynesworth et al., 1992a, 1995], as well as their secretory cytokine profile under standard growth conditions, or following stimulation by selected inductive molecules [Haynesworth et al., 1996]. These studies serve to establish the molecular phenotype of the MSC.

We have demonstrated that under appropriate tissue culture conditions, entire MSC populations can be directed into the osteogenic lineage. Aspects of the cellular and molecular events of this differentiation cascade have been described [Jaiswal et al., 1997], and the observations may be summarized as follows. MSCs exposed to optimal concentrations of dexamethasone, ascorbic acid, and β-glycerophosphate in vitro assume a cuboidal morphology, up-regulate alkaline phosphatase enzyme activity, express osteoblastic cell surface antigens, modulate the synthesis of osteocalcin mRNA in response to 1,25-$(OH)_2$ vitamin D_3 and dexamethasone, and deposit a mineralized hydroxyapatite extracellular matrix characteristic of osteoblasts and terminally differentiated osteocytes reproducibly within 16 days [Bruder et al., 1995a,b; Jaiswal et al., 1997]. The cellular events of this developmental phenomenon are similar to those described by other investigators using human bone marrow-derived cells [Cheng et al., 1994; Beresford et al., 1994; Rickard et al., 1996], although none of these investigators have reported the progression of such cells along as many lineages as has been demonstrated for human MSCs.

We have sought to examine the behavior of human MSCs (hMSCs) against the backdrop of stem cell biology and Leonard Hayflick's seminal work describing the limited proliferative capacity of normal human cells [Hayflick and Moorehead, 1961; Hayflick, 1965]. In summary, those studies demonstrated that fibroblastic cells derived from fetal lung tissue were capable of approximately 50 population doublings, while similar cells derived from adult lung lost their replicative potential after only 20 population doublings. Furthermore, cryogenic storage for as long as 27 years had no effect on the proliferation of these cells [Hayflick, 1989]. With this in mind, the goals of the current study were to determine the growth kinetics, self-renewing capacity, and the osteogenic potential of purified hMSCs during extensive subcultivation and following cryopreservation. By maintaining adult hMSCs in the log phase of growth, this study demonstrates that hMSCs have a relatively high replication capacity compared to other adult cell types. Despite the eventual loss of replication capacity with extensive passaging, the osteogenic potential of hMSCs is conserved throughout all subcultivations, and no differences in replicative or osteogenic potential are observed following cryogenic preservation.

MATERIALS AND METHODS

Materials

Dexamethasone (Dex), sodium β-glycerophosphate (β-GP), Percoll, penicillin/streptomycin antibiotic, DMSO, alkaline phosphatase diagnostic kit #85, and calcium diagnostic kit #587 were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM-LG (DMEM) was purchased from GIBCO (Grand Island, NY), L-ascorbic acid-2-phosphate (AsAP) from Wako Chemical (Osaka, Japan), and fetal bovine serum from Biocell Laboratories (Rancho Dominguez, CA) following an extensive testing and selection protocol [Lennon et al., 1996]. All other routine reagents used were of analytical grade.

Cell Preparation and Culture Methods

Bone marrow was obtained from nine human donors of various ages (Table I). Donors were either clinically normal or in remission from cancer, off protocol for chemotherapy or radiotherapy, and undergoing marrow harvest for future autologous bone marrow transplantation. Approximately 10 ml of unfractionated bone marrow was obtained by routine iliac crest aspiration after informed consent. Human MSCs were isolated from these marrow aspirates using methods modified from those described previously [Haynesworth et al., 1992a, 1996]. Briefly, 10 ml of marrow was added to 20 ml of control medium (DMEM containing 10% fetal bovine serum from selected lots), and centrifuged to pellet the cells and remove the fat layer. Cell pellets were then resuspended and fractionated on a density gradient generated by centrifuging a 70% Percoll solution at 13,000*g* for 20 min. The hMSC-enriched low density fraction was collected, rinsed with control medium, plated at 10^7 nucleated cells per 60 cm^2 dish in control medium, and cultured at 37°C in a humidified atmosphere containing 5% CO_2. Adherent hMSCs represent approximately 1 in 10^5 nucleated cells in this low density fraction. Nonadherent cells were removed on day 3 at the time of the first medium change, and fresh control medium was changed twice weekly thereafter. When culture dishes became near-confluent, cells were detached with 0.25% trypsin containing 1 mM EDTA for 5 min at 37°C.

For determining growth kinetics in primary cultures, the low density fraction described above was plated at 1.6×10^6 cells per 10 cm^2 dish in multiple dishes. On day 3, the culture medium was removed along with the nonadherent cells, and fresh control medium was added. Starting on day 3 and continuing each day until day 14, three dishes were used to quantify the number of adherent hMSCs as described below. Measurements were obtained on primary cultures from three different donors. For measuring growth kinetics of serially passaged cultures, primary hMSCs cultivated on 60 cm^2 dishes were trypsinized, counted on a hemacytometer, and a portion were replated at a density of 3×10^3 cells per cm^2 on sixty 10 cm^2 dishes, randomly divided in two groups. Following overnight attachment, cells were maintained for ten days with medium changes occurring daily in one group of dishes and twice weekly in the other group. Starting the day after plating (day 0) and continuing each day until day 9, three dishes from each group were used to determine cell number. The remaining dishes of primary hMSCs, which were not replated for the above growth kinetics assay, were subcultivated at a 1:3 split ratio onto new 60 cm^2 dishes. These cells were allowed to replicate until they were near confluent, at which time a portion of cells were again replated on sixty 10 cm^2 dishes. The remaining 60 cm^2 dishes were split 1:3, and serially passaged as above. This scheme of serial subcultivation, with growth kinetics assays at each passage, was continued until the cells became senescent. The number of adherent hMSCs in primary cultures was determined by counting the number of MSC colonies, each of which represent the progeny of a single hMSC [Haynesworth et al., 1992a,b]. Meticulous record-keeping of the number of cells present at the start and end of each passage facilitated calculation of the number of population doublings for any given passage number. Growth curves and population doubling calculations represent experiments performed using hMSCs from four marrow donors, and is presented as the mean plus or minus the standard deviation of the cell number measurements obtained for all three marrow donors combined.

TABLE I. Human MSC Donor Profile for each Study Component

Study Component	Donor Age	Clinical Condition	Gender
Osteogenesis & Cryopreservation	31	Normal	M
	39	Normal	M
Primary Culture	35	Acute Lymphoid Leukemia	M
Growth Kinetics	15	Renal Cell Carcinoma	F
	28	Normal	F
Passage Culture Growth Kinetics & Population Doubling Potential	43	Normal	F
	24	Breast Cancer	F
	3	Acute Myeloid Leukemia	M

Experiments to determine the osteogenic potential of hMSCs during extensive subcultivation and following cryopreservation were performed as follows. Aliquots of trypsin-released primary hMSC cultures were cryopreserved in FBS with 10% DMSO in liquid nitrogen, thawed 24 h later, tested for viability by Trypan Blue exclusion, and plated in three 60 cm^2 dishes at 5×10^3 cells per cm^2. A separate aliquot of the primary cells were identically detached and replated to perform direct comparisons of fresh versus cryopreserved hMSCs obtained from the same donor. When culture dishes from both groups (cryopreserved and unfrozen original) became near-confluent, cells were detached with trypsin (as described above), and replated in three 60 cm^2 dishes at a density of 5×10^3 cells per cm^2 for continued passaging and in 6-well tissue culture plates at 3×10^3 cells per cm^2 for in vitro osteogenic assays as described previously [Jaiswal et al., 1997]. This procedure for replating the cells was repeated with similar plating densities at each subculture when cells grown in the 60 cm^2 dishes became near-confluent. Detached cells were counted using a hemacytometer at the end of each passage in order to calculate the number of population doublings. The protocol for cell handling, subculture and osteogenic assays is diagrammatically presented in Figure 1. Cells were subcultured in this manner for up to 10 passages, and their in vitro osteogenic potential was measured at each passage in assays performed on days 4, 8, 12, and 16 as described below. For these in vitro osteogenic assays, the cells grown in 6-well plates were provided fresh control medium one day after plating (day 0) and subsequently grown in the absence or presence of an optimized mixture of Osteogenic Supplements (OS) (100 nM dexamethasone, 10 mM β-GP, and 0.05 mM AsAP) as previously described [Jaiswal et al., 1997]. Media changes were performed twice weekly, with a medium volume of 2 ml per well.

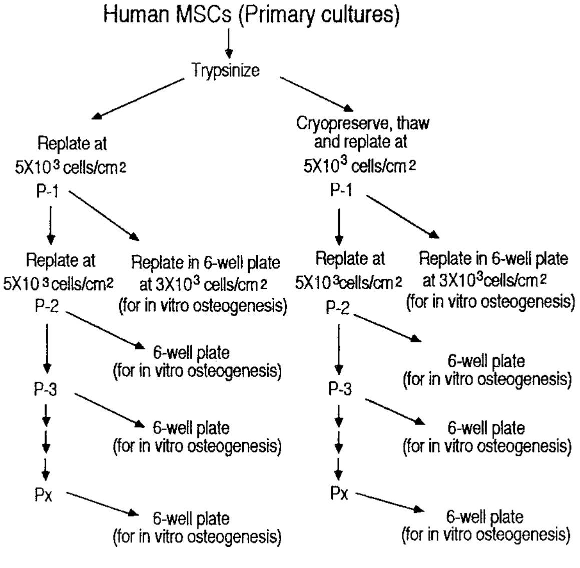

Fig. 1. Protocol showing cell cultivation for serial passaging of hMSC cultures. Primary hMSC cultures were trypsinized as described in Materials and Methods and half of the cells were replated in 60 cm^2 dishes at a density of 5×10^3 cells per cm^2. The remaining cells were cryopreserved overnight, thawed, and replated at a density of 5×10^3 cells per cm^2 in 60 cm^2 dishes. At 80–90% confluence, cells were trypsinized and replated in 60 cm^2 dishes (as described above) for subculturing and in 6-well plates at a density of 3×10^3 cells per cm^2 for in vitro osteogenesis assays. Cells used for in vitro osteogenesis assays are referred as being derived from passage 1 through X.

Cell Proliferation Assay

Cell proliferation was measured in triplicate cultures using a modification [Lennon et al., 1995] of the crystal violet dye-binding method [Westergren-Thorsson et al., 1991]. Cultures were rinsed twice with Tyrode's balanced salt solution, fixed with 1% glutaraldehyde (v/v) in Tyrode's for 15 min, rinsed twice with deionized water, and air-dried. Cultures were then stained with 0.1% crystal violet (w/v) in water for 30 min. After washing, crystal violet dye was extracted from the cells by 4 h rotary incubation at 25°C with 1% Triton X-100 (v/v in water). Absorbance of the resulting Triton extract was read at 595 nm on a microplate reader (Bio-Rad). Absorbance values were converted into absolute cell numbers based on established standard curves.

Alkaline Phosphatase Assay

Alkaline Phosphatase (APase) enzyme activity of the cell layer was measured in triplicate cultures by rinsing twice with Tyrode's balanced salt solution, and then incubating the cells with 5 mM *p*-nitrophenyl phosphate in 50 mM glycine, 1 mM $MgCl_2$, pH 10.5, at 37°C for 5 to 20 min. APase enzyme activity was calculated after measuring the absorbance of the reaction product formed, *p*-nitrophenol (pNP), at 405 nm on a microplate reader (Bio-Rad). Enzyme activity was expressed as nmol of pNP produced per min per dish.

Histochemical Analyses

Alkaline phosphatase histochemistry was performed for 1 h at 25°C as recommended by the manufacturer. During incubation, culture dishes were protected from drying and direct light. Dishes were rinsed with deionized water, and air-dried prior to viewing. Selected specimens were subsequently stained for mineral by the von Kossa method. Cell layers were fixed with 10% formalin for 1 h, incubated with 2% silver nitrate solution (w/v) for 10 min in the dark, washed thoroughly with deionized water and then exposed to bright light for 15 min.

Calcium Assay

Cell layers were rinsed twice with PBS and scraped off the dish in 0.5 N HCl. The calcium was extracted from the cell layers by shaking for 4 h at 4°C, then centrifuging at 1,000*g* for 5 min. The resulting supernatant was used for quantitative calcium determination according to the manufacturer. Absorbance of samples was read at 575 nm. Total calcium was calculated from standard solutions prepared in parallel, and expressed as µg per dish.

Statistics

Statistical analyses were performed using Student's *t*-test.

RESULTS

Growth Characteristics of Primary hMSC Cultures

Human MSCs were introduced into culture after enrichment from whole bone marrow cell suspension using a gradient composed of 70% Percoll. Human MSCs were allowed to attach to the surface of the plates for three days, at which time the culture medium was replaced with fresh medium. During days 2 to 5 of primary culture, adherent hMSCs can be observed as sparsely distributed individual spindle-shaped cells. In all of our previous studies, culture adherent hMSCs have been allowed to mitotically expand in primary culture with complete medium changes occurring twice weekly. When hMSCs in primary cultures expand to form colonies of several hundred cells that collectively cover 80–90% of the culture plate, the cells are passaged at a 1:3 dilution after being detached from the culture substratum with trypsin. The length of time from introduction of hMSCs into culture until their harvest for subcultivation into first passage is generally 12–14 days. In this study we measured the rate of division of hMSCs in primary cultures processed by our standard twice weekly medium change, and in cultures where the medium was changed daily.

The growth curves of hMSCs in primary cultures exhibit a lag phase of 6–8 days (Fig. 2). Upon visual observation on day 2, most of the adherent hMSCs were seen as individual, spindle-shaped cells that were sparsely distributed across the plate indicating that little mitotic expansion had taken place during the three day attachment period. In addition to the spindle-shaped hMSCs, round nonadherent cells were also observed. These nonadherent cells comprise the majority of cells originally seeded onto the plate. A small percentage of these nonadherent cells appear to loosely stick to the plates during the early days of culture without actually spreading out across the dish and becoming adherent to the substrate. Occasionally, these cells contribute to a transiently high background when cultures are analyzed for cell number by the crystal violet assay on days 2–4. By day 5 these cells are easily removed during the process of changing culture medium, and consequently, the background is reduced to undetectable levels. The lower limit of the crystal violet assay to accurately measure cell number is 1,000 cells as determined by standard curve. Because of this we stained plates with crystal violet dye and viewed adherent cells directly by phase contrast microscopy to complement the cell number measurement generated from the crystal violet colorimetric assay.

On day 3, a few small colonies of 4–8 hMSCs were observed, however, these early dividing cells represented only a small percentage of the total number of adherent cells (~500), which would eventually begin to divide and form larger

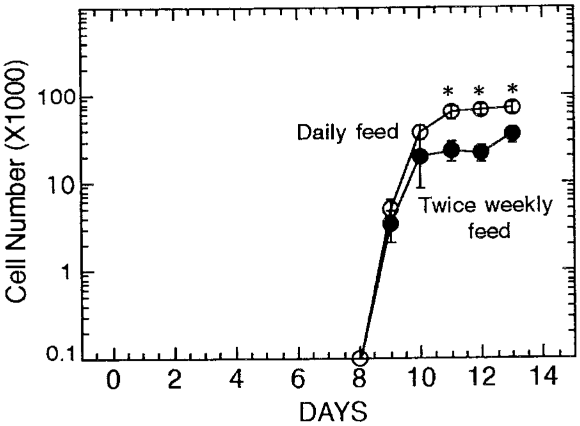

Fig. 2. Growth curves of hMSCs in primary culture. Percoll-fractionated hMSCs from whole bone marrow cell suspensions were seeded at 10^4 nucleated cells per cm^2 in 35 mm plates. hMSCs were allowed to attach for 3 days, after which the culture medium was changed on a twice weekly or daily schedule. Each day for 13 days, triplicate cultures were harvested for calculation of cell number as described in Materials and Methods. The results represent the mean cell number ± SD from one representative donor and experiment. $^*P < 0.05$.

colonies. Between days 4 and 6 the number of observable hMSC colonies increased, and a few large colonies could be observed resulting from the cells which had begun dividing by day 3. By day 8, the majority of hMSC colonies were established. Some of these colonies contained only 4 to 8 cells, while other colonies were very large and contained hundreds of cells. The pattern of formation of early, intermediate and late developing colonies was similar in both twice weekly and daily fed cultures. Colorimetric quantitation of hMSC cell number showed an exponential increase in cell number between days 8 and 10, followed by a plateau phase of slower cell growth from days 11 through 13. Although the media changing schedule did not appear to influence the initiation or growth of colonies during the lag phase, when hMSC cultures entered into log phase, daily fed samples generated a steeper growth curve and resulted in the formation of significantly more cells as compared to cultures fed twice weekly on days 10 through 13.

Growth Characteristics of Serially Passaged hMSC Cultures

Growth kinetics were measured at each passage beginning with passage 1 until culture senescence, which occurred between passages 10 and 15 depending on the donor. Cells were allowed to divide for 10 days with medium changes occurring twice weekly or daily. Figure 3 shows the growth curves obtained at passages 1, 4, 7, and 10 to illustrate the similarities and differences of hMSC growth characteristics in early, middle and late passaged cultures. At each passage, hMSC growth curves depict an initial lag phase of 24–36 h. This was followed by a log phase in which the hMSCs mitotically divided at exponential rates for 4–6 days, depending on the passage from which the cells were derived. The log phase was followed by a plateau phase where mitotic division continued through day 9 of culture, but at a slower rate.

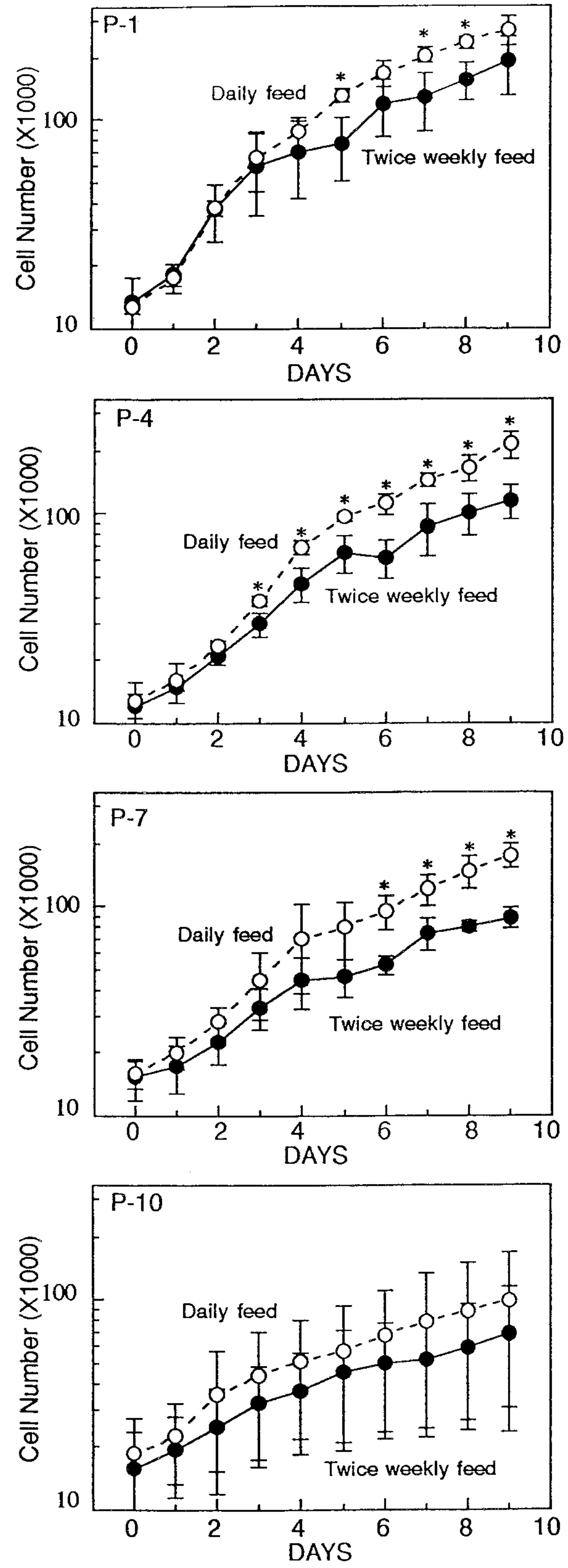

Fig. 3. Growth curves of hMSC cultures at passages 1, 4, 7, and 10. Cultures were seeded at 2×10^3 cells per cm^2 in 35 mm plates and fed on a twice weekly *(solid circles)* or daily *(open circles)* schedule. Each day for 9 days, one culture from each group was harvested for calculation of cell number as described in Materials and Methods. The results represent the mean cell number ± SD of three donor preparations. $^*P < 0.05$.

Human MSC cultures that were fed daily grew at faster rates and generated significantly more cells by the end of the 9 day growth period as compared to cultures supported by twice weekly medium changes (Fig. 3). For example, at passage 1, the number of cells in daily fed cultures was significantly greater on days 5, 7, and 8 ($P < 0.05$), and appreciably but not significantly higher on day 9 ($P = 0.12$) compared to twice weekly fed cultures. Daily fed cultures from passage 4 contained significantly more cells on days 3 through 9 ($P < 0.05$), while daily fed passage 7 cultures contained more cells on days 6 through 9 ($P < 0.05$), as compared to cultures fed twice weekly. By passage 10, however, as the cells approached the limits of their replicative potential, wide variability was observed in the number of cells on each day during the 9 day culture period for both daily and twice weekly fed cultures, resulting in no statistical differences in cell number.

With increasing passage number, the hMSC growth rates were slower and the number of cells generated by the end of 9 days in culture was reduced. Figure 4 illustrates that by passage 4, a decline in the number of cells generated during the 9 day culture period was observable. For example, 9 days after replating cells derived from passages 1, 4, 7, and 10, the mean yields for twice weekly fed cultures were 2.7×10^5, 1.1×10^5, 0.88×10^5, and 0.68×10^5 cells, respectively. This reduction did not result in a statistically significant fewer number of cells in twice weekly fed cultures at any time point in passage 4 (Fig. 4a). However, in daily fed cultures at this passage, proliferation was compromised between days 5 and 8 ($P < 0.05$) when compared to the growth of cells derived from passage 1. At passage 7 and beyond, the growth of cells had waned in comparison to passage 1 cultures at nearly every time point beyond day 5 ($P < 0.05$). This slowing of cell proliferation as a function of increasing passage number was independent of the feeding schedule used to maintain the cells.

Self-Renewal Capacity of hMSCs

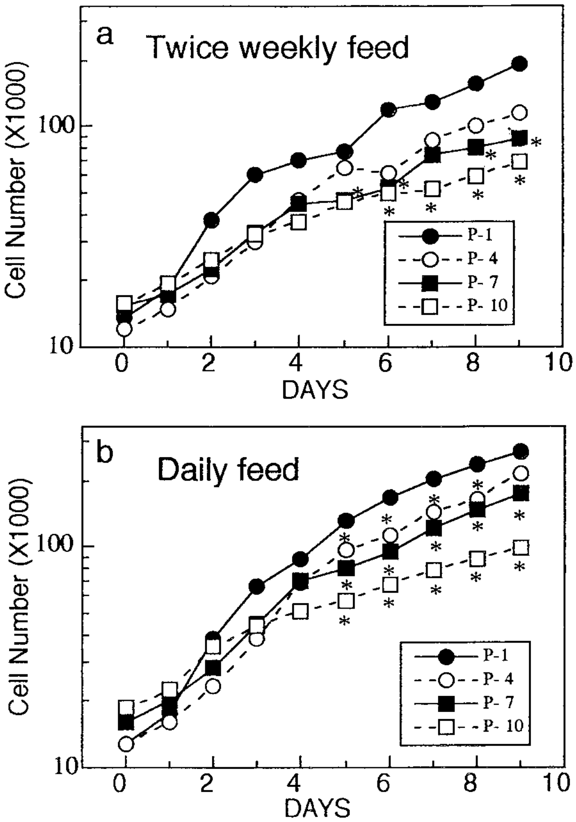

Fig. 4. Growth curve comparisons of hMSCs at passages 1, 4, 7, and 10. At each passage, cells were seeded at 2×10^3 cells per cm^2 in 35 mm plates and fed on a twice weekly (**a**) or daily (**b**) schedule. Each day for 9 days, one culture from each group was harvested for calculation of cell number as described in Materials and Methods. The results represent the mean cell number ± SD of three donor preparations. *$P < 0.05$ (compared to P-1 cultures).

The total population doubling potential was calculated from the number of population doublings determined for each passage until the time of replicative senescence from four different donors. For primary cultures, the initial number of hMSCs that attached to the plate and divided to form colonies was estimated as the average number of colonies which form in primary culture based on our colony count database of over 50 donors (data not shown). All other determinations represent the actual number of hMSCs counted at the end of each passage prior to introducing the cells back into culture for the next passage. The mean cumulative population doublings for the four donor hMSC preparations was 38 ± 4 (Fig. 5). On average, 11.2 population doublings took place in primary culture, accounting for 29.4% of the mean cumulative number of population doublings. The average starting number of hMSCs was 500 per dish, and the average final number at the end of primary culture was 6.1×10^5. For passages 1–10, the average number of population doublings at each passage was about 2, which was expected since the cultures were

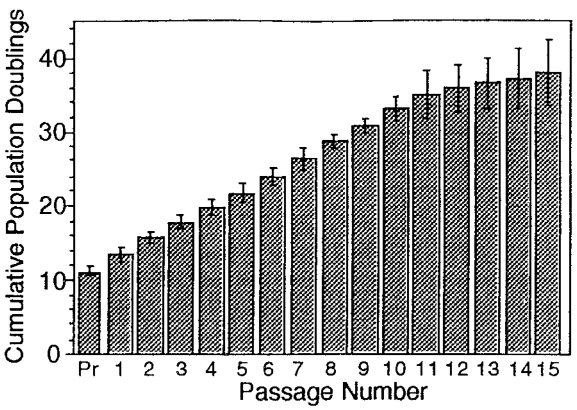

Fig. 5. Population doubling potential of hMSCs. Cumulative population doublings were calculated from the initial number of developing hMSC colonies and the number of hMSCs harvested at each passage. Results are presented as the mean cumulative populating doublings ± SD of hMSCs derived from four different donors. Primary cultures undergo approximately 11 population doublings prior to subcultivation, whereas passaged cells are plated at densities which result in approximately 2 population doublings per passage.

replated when they became 90–95% confluent at each passage. After tenth passage, the number of population doublings declined as senescence was reached in the different donor preparations. By passage 12, hMSCs in two of the four donor preparations had stopped dividing, while cells in the other two cultures continued to divide very slowly and were passaged prior to achieving 90% confluence to determine if replating would stimulate further cell division. As observed in Figure 5, few additional population doublings were generated by cells subcultured from passages 12 through 15.

Continuous Subculturing of hMSC Cultures

When primary cultures of hMSCs were subcultivated, cells attached uniformly to the culture dishes at approximately 30% confluence and reached 80–90% confluence in 5 days, at which time cells were subcultured again. As passage number increased, the time between initial plating and subsequent subculturing increased from 5 days to approximately 10 days by tenth passage. (Fig. 4a). Uniform attachment and spindle-shaped morphology of hMSCs was observed on cells at every passage until the hallmarks of cellular senescence appeared during the final subculture. These features include cessation of mitotic activity, accumulation of cellular debris and stress fibers, a broad flattened morphology, and ultimately, total degeneration of the culture. Retention of the MSC phenotype following serial passaging has previously been confirmed on all cells by positive cell surface immunostaining with MSC-specific monoclonal antibodies [Haynesworth et al., 1992a].

Induction of Osteogenesis by OS in hMSC Cultures

As described previously [Jaiswal et al., 1997], MSCs cultured with OS undergo a dramatic change in cellular morphology from that of spindle-shaped to cuboidal, which is accompanied by an increase in APase activity and hydroxyapatite mineral deposition. This increase in APase activity and mineral deposition was consistent for all MSC preparations regardless of whether they were extensively subcultured and/or cryopreserved, and is illustrated by one representative sample in Figure 6a. A significant increase in APase activity was observed after 4 days of OS treatment with maximal activity occurring on day 12, followed by a decline by day 16. This late decrease in APase activity of OS cultures is reproducible, and correlates with increasing mineral deposition and terminal differentiation of cells into osteocytes. Although the timing of peak APase activity varies from day 8 through 12 for different donors and samples, with peak activity ranging from 7.5 to 75 nmol pNP/min/10^6 cells, OS universally stimulates APase expression in MSC cultures that eventually declines by day 16 [Jaiswal et al., 1997]. These cultures were also studied for their ability to elaborate mineralized extracellular matrix when grown in the presence or absence of OS. No calcium deposition was detected either by Von Kossa staining or the sensitive colorimetric quantitative calcium assay in control MSC cultures. As shown in Figure 6a, MSCs grown with OS showed a significant calcium deposition as early as day 8 (1.32 ± 0.05 μg/dish), with a further increase on days 12 (16.26 ± 2.94 μg/dish) and 16 (31.18 ± 1.52 μg/dish). As illustrated in Figure 6b, treatment with OS also significantly increased cell number on days 8, 12, and 16. The results presented here and below reflect experiments performed with MSCs derived from one donor, although similar results were obtained with MSCs derived from other donors which were cryopreserved and/or extensively subcultured.

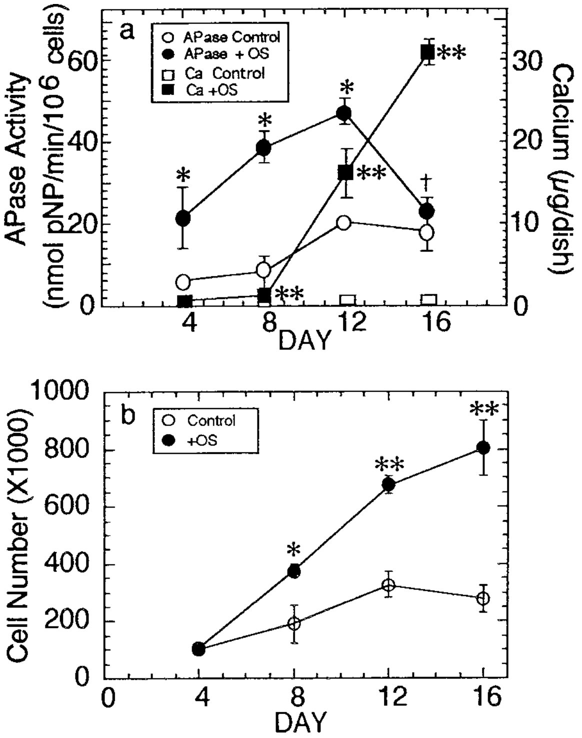

Fig. 6. APase activity, calcium deposition (a), and cell proliferation (b) in hMSC cultures grown in control or OS medium on days 4, 8, 12, and 16. Samples were harvested at the indicated days, and APase activity, calcium deposition and cell number were determined as described in Materials and Methods. The results represent the mean ± SD of triplicate cultures of one representative experiment derived from first passage cells of one donor. $^*P < 0.05$; $^{**}P < 0.001$ (compared to control).

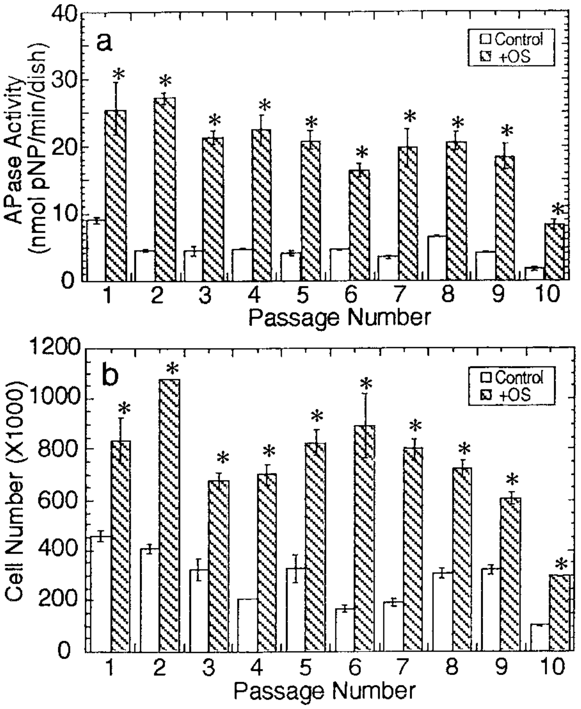

Fig. 7. APase activity on day 8 (a) and cell proliferation on day 12 (b) as a function of passage number in control or OS medium. Cells were seeded at 3 × 10^3 per cm^2 and switched to OS medium the following day. Cultures were assayed for APase and cell proliferation as described in Materials and Methods. The results represent the mean ± SD of duplicate cultures of one representative donor. $^*P < 0.05$ (compared to control).

Osteogenic Differentiation and Cell Proliferation as a Function of Passage Number in Control and OS-Treated hMSC Cultures

Figure 7 illustrates the APase activity and cell proliferation on days 8 and 12, respectively, of serially subcultured hMSCs grown in the absence and presence of OS. Relatively little variability occurred in the low basal APase activity (range, 3.42 ± 0.29 to 8.91 ± 0.49 nmol pNP/min/dish) of these cells even after subculturing nine times. Human MSC cultures treated with OS showed a significant increase in APase activity on day 8 in cells derived from passages 1 through 10 (Fig. 7a). Interestingly, the lowest APase activity was observed in cells derived from passage 10 in this donor (>30 population doublings), which parallels the point where mitotic activity decreased and cells began to degenerate in a manner similar to Hayflick's Phase III senescence [Hayflick, 1965]. Similar to control cultures, OS cultures derived from passage 10 had the lowest absolute value of APase activity. Although the absolute level of APase activity on day 8 varied from one passage to another, a 3–5-fold increase in APase activity was invariably observed in these serially passaged, OS-treated hMSCs. Furthermore, despite that fact that cells derived from tenth passage expressed the lowest level of APase activity in both control and OS-treated cultures, the fold stimulation observed in OS-treated cultures was comparable to that obtained in cultures derived from passages 1 through 9. Additional evidence supporting the osteogenic differentiation of these extensively subcultured MSCs is provided in Table II, which indicates the maximum fold stimulation of APase activity per cell for each passage.

Figure 7b depicts cell number at day 12 in the hMSC cultures derived from sequential passages. The cell number in control cultures from passage 2 through 9 was approximately 2–4 × 10^5 cells on day 12, or about 10-fold greater than the number of cells originally seeded on the dish, and clearly represents cells in the

TABLE II. Cell Proliferation and Osteogenic Potential of hMSCs as a Function of Passage Number

Cell Preparation	Number of MSCs	Fold Expansion of MSCs	Maximum Fold Stimulation of APase by OS
Initial Isolation	2200	0	N/A
Primary Culture	16×10^6	7.3×10^3	N/A
1st Passage	53×10^6	24×10^3	9.1
2nd Passage	176×10^6	81×10^3	9.1
3rd Passage	586×10^6	270×10^3	10.5
4th Passage	1.9×10^9	901×10^3	17.7
5th Passage	6.5×10^9	3×10^6	17.5
6th Passage	22×10^9	10×10^6	17.2
7th Passage	72×10^9	33×10^6	14.5
8th Passage	241×10^9	110×10^6	18.7
9th Passage	803×10^9	370×10^6	9.7
10th Passage	2.7×10^{12}	1.2×10^9	19.7

plateau phase of growth as described above. Furthermore, the decline in cell proliferation as a function of passage number is reproduced in this series of experiments, with cell numbers from assays at passages 7 and 10 significantly ($P < 0.001$) lower than those from passage 1. Interestingly, although the baseline rate of cell division decreased as a function of passage number, the addition of OS to these MSCs caused a characteristic and significant increase in cell number at every passage tested ($P < 0.05$). Visual inspection as well as cell counting of the MSC cultures derived from tenth passage revealed that at this stage of subculture for this MSC donor, the rate of cell proliferation decreased markedly. Additional subcultivation and osteogenic assays were not performed beyond tenth passage since the cells had become senescent and degeneration ensued. The relatively low values for APase activity of control and OS-treated cultures derived from tenth passage, when expressed as nmol pNP/min/dish, are a direct result of the low cell numbers present at day 8 in extensively subcultured cells. The cell proliferation data from this hMSC donor, with respect to the self-renewing capacity of approximately 30 population doublings, lies within the range detailed in experiments presented in Figure 5.

Spindle-shaped hMSCs at the start of cell culture in each passage became cuboidal within 48 hours of OS treatment. On day 4, control cultures contained only few (<1%) APase-positive cells, whereas approximately 30–40% of cells were APase-positive when cultured in OS medium. By day 16, control cultures grew as a uniform sheet of cells in a whirling pattern with about 5% APase positive spindle-shaped cells. OS-treated cultures displayed strong APase staining in approximately 90% of all cells after 8 days of treatment for all passages. As the cells grew to confluence by day 12, multilayered bone-like nodular aggregates were observed throughout the dish when cultured in OS medium. After 16 days in OS medium, the cells reproducibly formed a mineralized matrix. Figure 8 illustrates the control and OS cultures from early, mid, and late passages when stained for APase and mineral after 16 days of culture. The critical feature in these photomicrographs is the observation that the morphologic and developmental changes which hMSCs undergo in response to OS do not vary as a function of the passage number from which the starting cells were derived. Additionally, the quantitative changes in APase activity occurring on days 4–16 of control and OS-treated samples from all subcultures (data not shown) duplicate the phenomenon described in Figure 6a using cells derived from first passage. That is, one can not distinguish cells derived from first passage through ninth passage based on the biochemical and morphologic changes in response to OS-induced osteogenic differentiation.

Osteogenic Differentiation and Cell Proliferation in Serially Passaged hMSC Cultures Following Cryopreservation

In an effort to determine whether the self-renewing capacity and/or osteogenic potential of hMSCs preserved in liquid nitrogen is similar to that of the original unfrozen cultures, we performed the above series of analyses on cryopreserved cultures in parallel with unfrozen cultures as outlined in Figure 1. In our hands, cell recovery following thawing routinely exceeds 95%, and is consistent with the viability of numerous cell lines and hybridomas routinely maintained in our laboratory. The results of that comparison showing control and OS-treated cultures are presented in Figure 9. Figure 9a shows that basal APase activity on day 8 in both cryopreserved and non-cryopreserved cultures were not significantly different from each other ($P > 0.05$), except for passages 1, 2, and 4. Basal APase activity in cryopreserved and non-cryopreserved cultures ranged from

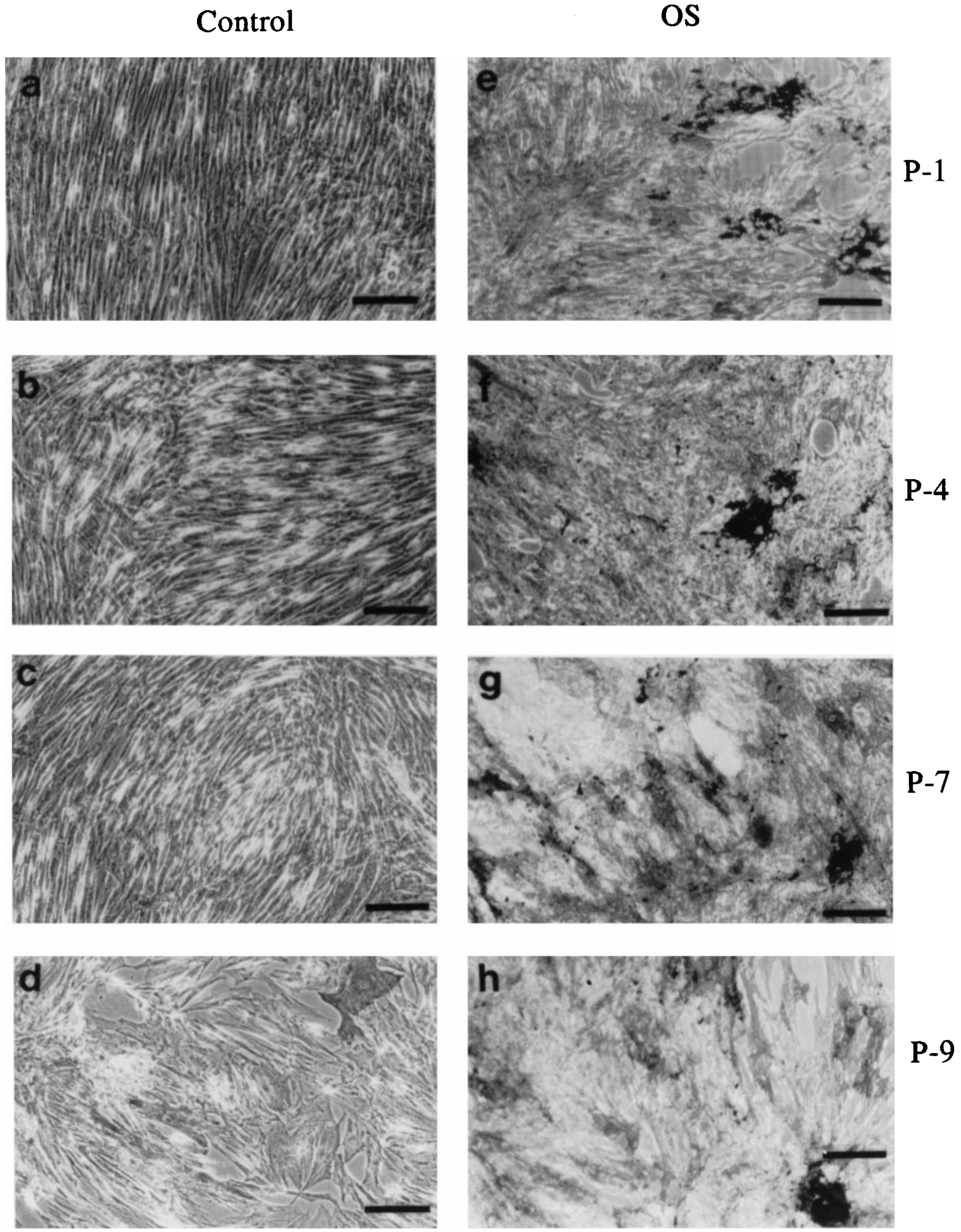

Fig. 8. Effect of OS on cell morphology, APase expression, and mineral deposition in 16 day hMSC cultures derived from first, fourth, seventh, and ninth passages. All specimens were stained by the APase and von Kossa histochemical techniques as described in Materials and Methods. Phase contrast microscopy demonstrates the spindle-shaped morphology of uniformly dense, APase negative control MSCs through seventh passage (**a–c**), whereas cells in ninth passage (**d**) begin to display a broadened, flat morphology. By contrast, cells grown with OS are polygonal, APase positive, and exhibit nodular aggregates with von Kossa staining mineral (**e–h**). The formation of nodular aggregates in OS-treated cells frequently results in the exposure of bare tissue culture plastic visible in e–h. Scale bar = 200 μm.

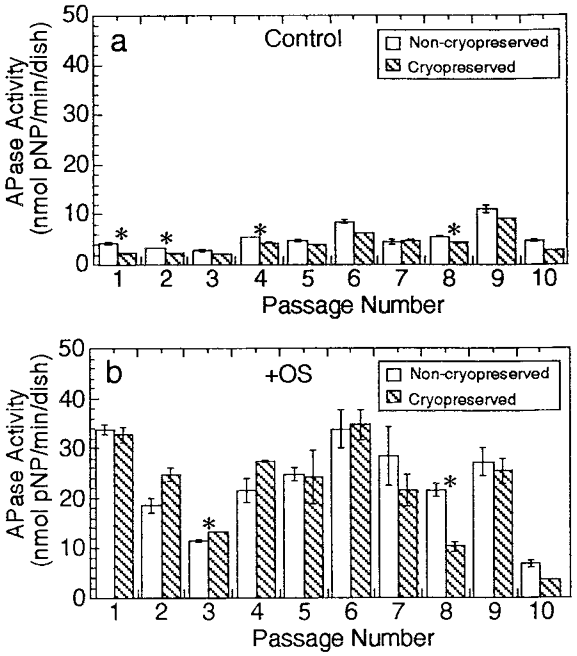

Fig. 9. APase activity as a function of passage number and cryopreservation in control (a) and OS-treated (b) hMSC cultures on day 8. Cells which were cryopreserved for 24 h and noncryopreserved samples were seeded at 3 × 10^3 per cm^2 and switched to OS medium the following day. On day 8, cultures were assayed for APase as described in Materials and Methods. The results represent the mean ± SD of duplicate cultures of one representative donor. $^*P < 0.05$ (compared to noncryopreserved cells).

2.20 ± 0.03 to 9.29 ± 0.04, and 2.88 ± 0.25 to 11.22 ± 0.83 nmol pNP/min/dish, respectively. Figure 9b illustrates that treatment of cryopreserved, serially passaged hMSC cultures with OS markedly enhanced the APase activity as previously described for unfrozen cultures. Importantly, although the absolute value of APase activity varies from passage to passage, the phenomenon of a 3–5-fold increase in APase activity of OS-treated cells derived from cryopreserved cultures exactly duplicates the results obtained from experiments using cells from any given passage which were never frozen.

As illustrated in Figure 10a, the growth potential of MSCs following cryopreservation is not significantly different ($P > 0.05$) than that of cells which were never frozen. Continued growth was observed for ten passages, until division began to slow and cells eventually degenerated as described above. Again, in this donor, the cell number at day 12 in samples derived from passage 7 and 10 was significantly ($P < 0.005$) lower than the cell number in samples derived from passage 1. Furthermore, the characteristic mitogenic response of MSCs to OS presented in Figure 7b is retained following cryopreservation (Fig. 10b). Here again, the results obtained with cryopreserved cells mirror the observations made with cell preparations which were never frozen.

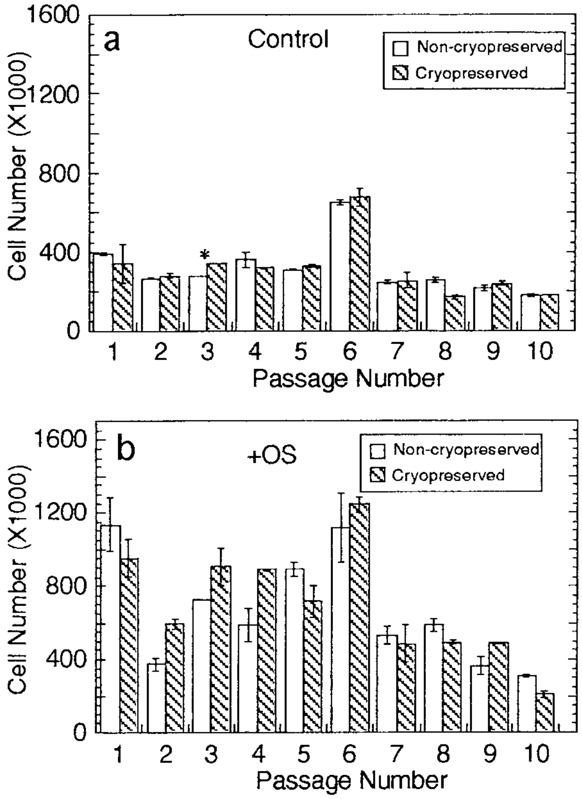

Fig. 10. Cell proliferation as a function of passage number and cryopreservation in control (a) and OS-treated (b) hMSC cultures on day 12. Cells which were cryopreserved for 24 h and noncryopreserved samples were seeded at 3 × 10^3 per cm^2 and switched to OS medium the following day. On day 12, cultures were assayed for cell proliferation as described in Materials and Methods. The results represent the mean ± SD of duplicate cultures of one representative donor. $^*P < 0.05$ (compared to noncryopreserved cells).

DISCUSSION

In the present study, we have provided further characterization of a population of cells derived from human bone marrow which are found in very low abundance, possess high self-renewal capacity, and retain their undifferentiated phenotype through senescence, or until such time that inductive cues direct the cells into a restricted terminal differentiation pathway. These cells are referred to as human mesenchymal stem cells (MSCs) because they are known to give rise to multiple mesodermal tissues including bone [Haynesworth et al., 1992b; Bruder et al., 1995b; Jaiswal et al., 1997] cartilage [Lennon et al., 1996; Johnstone et al., 1996], fat [Pittenger et al., 1996], and hematopoietic supportive stroma [Majumdar et al., 1995]. We report here on the growth kinetics of purified, culture-expanded human MSCs, and that their behavior is consistent with other normal human diploid cells. We present additional data which demonstrates full retention of their osteogenic potential following cryopreservation and extensive subculture. Together, these investigations support the characterization of this population of cells as stem cells.

MSC cultures are initiated as primary cultures of fibroblastic cells which grow out of marrow cell suspensions by selectively attaching to tissue culture plastic and dividing to form colonies. The process of colony growth from the newly adherent fibroblastic cell population is not uniform. Instead, some cells appear to quickly give rise to colonies soon after adherence to the culture dish, whereas other cells do not yield colonies until several days of culture. The rate of cell division is also variable with some colonies enlarging rapidly after the initial cell division, while other colonies expand slowly. This bimodality in the rate of colony formation is well documented for fibroblastic cells in cultures derived from clonal and mass cell origin [Martin et al., 1970; Martinez et al., 1978; Matsumura et al., 1979; Mitsui and Schneider, 1976]. Fibroblastic cells from fetal and adult skin that replicate rapidly to form large colonies have been shown to possess higher population doubling capacity than cells that divide slowly and form small colonies [Smith et al., 1978]. Similar observations were made by Mets and Verdonk [1981] in their study of in vitro aging characteristics of human bone marrow stromal cells. These investigators distinguished two adherent cell types in the primary cell population. Type I cells were spindle-shaped and rapidly dividing, while Type II cells were broader and divided slowly if at all. The majority of cells in primary cultures were Type I cells, however, during subcultivation the appearance of Type II cells formed an increasing fraction of the total cell population. These observations led to the interpretation that Type I cells are progenitor cells which gradually give rise to Type II cells through tangential or asymmetric cell division, and that Type II cells represent a terminally differentiated state of nondividing cells.

Our observations also indicate that primary hMSC cultures contain cells with varying rates of division. During early subcultivations, spindle-shaped, rapidly dividing cells generate a near homogeneous population. However, after 5–6 passages without differentiation, the morphology of the cells becomes heterogeneous with broad cells distributed among the spindle-shaped cells. This likely occurs as some daughter cells, generated from the asymmetric cell division of spindle-shaped cells, acquire a broad morphology and reduced replication capacity, while other daughter cells retain the spindle-shape and high replication capacity. Differences in population doubling potential among human fibroblastic daughter cells from derived from the division of a single cell have been documented to differ by as many as eight population doublings [Smith and Whitney, 1980]. The source of this heterogeneity is not clearly understood. Since the majority of hMSCs are broad in morphology by 10th passage, these observations are consistent with those of Mets and Verdonk [1981] who noted that spindle-shaped rapidly dividing fibroblasts give rise to broad-shaped fibroblasts with diminished replication capacity. Our observations do not support the interpretation by Mets and Verdonk that the nondividing broad cells represent a terminally differentiated population. Instead these broad cells, like the spindle-shaped cells of early passage, do not express osteogenic traits under control medium conditions, but indeed retain the capacity to differentiate along the osteogenic lineage when exposed to OS, and to also increase their rate of division in response to OS.

At each passage, hMSCs followed a normal growth curve consisting of a lag phase followed by a log phase of exponential cell growth, ending with a plateau phase in which the growth rate declined. In daily fed cultures, the length of the exponential growth phase is similar to that of cultures fed twice weekly. However, the steeper slope of the growth curves during this phase results in a higher density of cells before the rate of growth begins to decline. The rate of growth and level of density-dependent inhibition of further growth is likely influenced by the concentration of serum-derived growth factors in the culture medium. In cultures fed twice weekly, the concentration of mitogenic factors may become rate-limiting as the cells metabolize the culture medium, whereas cells maintained in daily fed cultures are exposed to a consistently high concentration of serum-derived factors. As the number of population doublings increases, the rate of population growth declines. Studies on other types of fibroblastic cells have shown this process to involve a percentage of cells leaving the cell cycle at each successive division, and resulting in a lower net percentage of cells in the population which continue to divide [Martin and Sprague, 1970; Martinez et al., 1978; Matsumura et al., 1979; Mitsui and Schneider, 1976; Mets and Verdonk, 1981]. Data from these studies do not support the opposing hypothesis that the decline in population growth rate is due to a uniform decline in the rate of cell division throughout the cultures [Karatza et al., 1984].

Regardless of the tissue source, fibroblastic cell growth follows a predictable sequence with increasing age in vitro, eventually resulting in loss of replicative potential [Hayflick and Moorehead, 1961; Hayflick, 1965]. Likewise, hMSCs possess many of the growth characteristics of other types of fibroblasts in culture. Importantly, the mean cumulative population doublings of marrow-derived hMSCs from the adult donors used in this study was 38 ± 4. This number is greater than the average of 20 population doublings which occurs in adult lung fibroblasts [Hayflick, 1965] and keratinocytes [Rheinwald and Green, 1975]. However, it is less than the 50 population doublings obtained from fetal lung fibroblasts [Hayflick, 1965], and the 70 population doublings observed with human umbilical vein endothelial cells [Thornton et al., 1983]. These data suggest a population doubling potential of hMSCs which is intermediate to what has been reported for other human cells derived from either embryonic or adult tissues. Since the population doubling potential has been used widely as an index of biological age, the higher population doubling potential of hMSCs suggests that these cells are of a younger biological age than other normal adult cells, and as such, may provide an in situ source for mesenchymal progenitor cells well into adulthood.

As with other culture adherent cells, the mechanism for the loss of population doubling potential after a finite range of population doublings is not well understood. Several mechanisms for the cause of replication senescence have been put forth, including the accumulation of genetic damage [Szilard, 1959; Orgel, 1973], the shorting of telomeres [Harley et al., 1990] and the activation of tumor suppressor [Sager, 1989] or senescence genes which code for proteins that block the cell cycle [Wang et al., 1994]. In addition, replication senescence has been described as a type of terminal cell differentiation [Bell et al., 1978]. Future studies will be needed to determine which of these mechanisms are involved in the replicative senescence of hMSC and whether the replication senescence of all types of fibroblasts are controlled by the same mechanisms.

The events which occur during osteogenic differentiation of hMSCs, that is, commitment, lineage progression, and elaboration of a mineralized matrix, have been documented previously [Bruder et al., 1995b; Jaiswal et al., 1997]. One of the hallmarks of this process is the significant elevation in APase activity which peaks between days 8 and 12 of culture, depending on the donor, and the passage from which the cells were derived. Experiments testing the osteogenic potential of hMSCs as a function of passage number clearly indicate that such elevations in APase activity in response to OS consistently occur in the same high proportion of cells derived from every passage (Fig. 7a). The formation of characteristic mineralized nodular aggregates throughout the culture dish was also observed in specimens from every passage. Furthermore, the fact that the basal APase activity remains low in control samples derived from extensively passaged cells supports our assertion that prolonged tissue culture does not result in the selective outgrowth of a subpopulation of MSCs with a predilection to become osteogenic. Although comprehensive studies showing other mesenchymal developmental potentials of hMSCs at every passage from a single donor have not been performed, adipogenesis [Pittenger et al., 1997], chondrogenesis [Johnstone et al., 1996; Lennon et al., 1996] and stromagenesis [Majumdar et al., 1995] are routinely observed in preparations derived from first through fourth passage. Together, these data refute the possibility that serial subcultivation of hMSCs favors retention of osteogenic potential at the expense of other developmental pathways. Beyond the osteoinductive effect which OS has on hMSCs, OS also acts as a mitogen (Fig. 6b). Figure 7b illustrates that this mitogenic response of hMSCs to OS is also conserved following serial subculture. These data, together with studies of in vivo osteogenesis of passaged hMSCs [Haynesworth et al., 1992b], demonstrate that the osteogenic potential and mitogenic response of hMSCs to OS following extensive subculture is not diminished. Therefore, it appears that the replicative senescence which occurs in late passage is not due to terminal differentiation of Type II cells, as suggested by Mets and Verdonk [1981]. Certain techniques relating to the way the hMSCs are initially isolated, grown, and subcultivated undoubtedly contribute to the perpetuation of these cells without lineage progression, not the least of which is the selection of an appropriate lot of fetal bovine serum [Lennon et al., 1996].

We also sought to determine whether the process of cryopreservation affected either the proliferative capacity of these cells, or their developmental potential. Such cryogenic manipulation is particularly interesting in view of the fact that other investigators use cryopreservation in their technique for selecting and isolating mesenchymal stem cells from tissues other than bone marrow [Young et al., 1991, 1993, 1995]. Since virtually all cryopreserved cells were viable, the results obtained from experiments with these cells cannot be construed to reflect the activity of a subpopulation of the originally frozen hMSCs. As presented in Figure 9a, cryopreserved cells which are subcultured in parallel with cells never frozen possess the same low basal APase activity. Importantly, in response to OS (Fig. 9b), the increase in APase activity of cryopreserved hMSCs mirrors OS's effect on noncryopreserved cells and, for the most part, is not statistically different. That is, in passages where the absolute activity is either relatively low or high, both fresh and frozen cells from the same donor behave identically. The same phenomenon holds true with respect to the proliferation and mitogenic response of cells cultured in the absence or presence of OS (Fig. 10). These observations suggest, as others have previously shown [Hayflick and Moorehead, 1961; Hayflick, 1965, 1989], that cells have a “memory,” which is primarily influenced by their duration and manipulation in tissue culture rather than their calendar age or the process of cryopreservation. To be sure, cells which were frozen and stored for as long as 27 years have been shown to behave the same as the starting cells which were never cryopreserved [Hayflick, 1989]. Nevertheless, it is possible that our 24 h “cryopreservation” protocol may not be indicative of results obtained with MSCs stored for decades.

In view of the above observations, it may now be possible to explore clinical alternatives using self cell therapies which have never before been available [Bruder et al., 1994; Caplan and Bruder, 1996]. Since the reduction of bone mass in osteoporosis, normal aging, and a variety of other diseases has been linked with a diminution in the number and activity of marrow-derived osteoprogenitor cells [Tabuchi et al., 1986; Tsuji et al., 1990; Egrise et al., 1992; Quarto et al., 1995; Kahn et al., 1995; Bergman et al., 1996], one idealized therapeutic goal would be to rejuvenate the supply of osteogenic progenitor cells, or MSCs. The present study demonstrates that MSCs can be cryopreserved and expanded over one billion fold ex vivo without a loss in their osteogenic potential (Table 2). Therefore, for the treatment of diseases based on an inadequate supply of MSCs, we would propose the periodic administration of cryopreserved, autologous MSCs based on the safe intravenous infusion protocol recently reported [Lazarus et al., 1995]. Successful application of this technology for the treatment of metabolic, genetic, and structural skeletal abnormalities shall serve to guide aspects of our future efforts.

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