The Effect of Extended First Passage Culture on the Proliferation and Differentiation of Human Marrow-Derived Mesenchymal Stem Cells

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ABSTRACT

Human marrow-derived mesenchymal stem cells (hMSCs) have been investigated for more than 20 years. They have been shown to be therapeutic in a number of animal models and are currently in use in more than 200 clinical trials, thus documenting their importance in the field of translational medicine. Standard protocols for the passage and collection of hMSCs involve trypsinization of preconfluent cultures. This practice is based, at least in part, on concerns that the multipotency of these cells would be diminished if the cultures became confluent. To test this concern, hMSCs were isolated and maintained in standard culture conditions in primary culture and were then subcultured after 2 weeks. The resulting first passage cultures were divided into two groups: those that were subcultured at the normal frequency, usually at 7 days for each passage (referred to as standard conditions [SC]), and those that were maintained for up to 53 days without being further subcultured (extended first passage [EFP]). At the end of the second passage and each of five subsequent subcultures for cells in SC (i.e., through passage 7), complementary EFP cultures were also trypsinized. Cells from each group were counted, resuspended in serum-free medium, and assayed to determine the ability of the cells to differentiate along osteogenic, chondrogenic, and adipogenic lineages. Cells in SC experienced an average of 27 population doublings through seven passages, whereas hMSCs in EFP achieved approximately 16 population doublings after 34 days but demonstrated very little increase in cell number after that time. The ability of hMSCs in EFP to produce bone in ceramic cubes implanted subcutaneously in immunocompromised mice and to differentiate into cartilage in pellet or aggregate culture was at least equivalent to that of the cells in SC through seven passages, whereas the capacity of the EFP hMSCs to produce lipid droplets in adipogenic conditions was maintained but was diminished relative to that of SC cells.
standard protocol, in which the cells are subcultured weekly after primary culture, and as extended first passage (EFP) cultures. EFP cultures were given a complete change of medium twice weekly, but they were not subcultured as was done for standard cultures. However, each time the standard cultures were trypsinized and subcultured, hMSCs from a limited number of EFP cultures were also released with trypsin-EDTA. The two parallel groups of cells from the same donor were counted to determine growth dynamics, and they were tested in assays to determine how the two treatment methods had affected their ability to differentiate along osteogenic, chondrogenic, and adipogenic pathways.

**Materials and Methods**

**Cell Isolation**

Cultures of human marrow-derived mesenchymal stem cells were established as previously described [14, 22]. For each hMSC preparation, a small volume of bone marrow was aspirated from the posterior superior iliac crest of a healthy donor, whose informed consent had been obtained under University Hospitals of Cleveland institutional review board protocol number 09-90-195. The bone marrow sample was resuspended in Dulbecco’s modified Eagle’s medium with low glucose (DMEM-LG; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, http://www.invitrogen.com) from a lot selected for its ability to support the proliferation and differentiation of hMSCs [23]. After the marrow suspension was centrifuged (450 g), the resulting pellet was resuspended in a small volume of medium and deposited at the top of a tube of Percoll (density, 1.03 to 1.12 g/ml; Sigma-Aldrich); the tube was then centrifuged at 480 g. Centrifugation concentrates the red blood cells at the bottom of the tube while retaining the nucleated cells at the top of the Percoll gradient. The top 10–14 ml of the gradient was collected, transferred to a separate 50-ml polystyrene tube, rinsed with additional serum-supplemented medium, and centrifuged again to remove the residual Percoll. The resulting pellet was resuspended in a small volume of serum-containing medium, and the nucleated cells were counted with a hemacytometer. Nucleated cells were seeded at a density of 180,000 per cm³ in standard culture vessels, and the medium was changed every 3–4 days.

**Subculture**

After approximately 14 days (before confluence), the cells were rinsed with Tyrode’s salt solution (Sigma-Aldrich) and then exposed to trypsin-EDTA (0.25% trypsin, 4 mM EDTA; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for 5 minutes. The action of the trypsin was arrested by the addition of bovine calf serum (Gemini Bio-Products, West Sacramento, CA, http://www.gembio.com), and the cell suspension was collected and centrifuged at 400 g for 5 minutes. The resulting cell pellet was resuspended in a small volume of serum-supplemented medium, and the cells were counted and seeded as first passage cultures at 4,500 cells per cm². At this point, the resulting first passage cultures were divided into two groups: those that would be subcultured, in the manner described above, before the cells became confluent (approximately every 7 days) (standard conditions [SC]), and a second group that would not be subcultured again (EFP) except for a small number of culture flasks that were trypsinized each time the cells in SC were passaged and then tested to assess their differentiation potential. Medium for cultures in both groups was changed every 3 or 4 days.

**MSC Proliferation**

MSCs in SC were counted using a hemacytometer at the end of each passage. At the same time, a small number of flasks containing cells in EFP were also trypsinized and counted. Accordingly, as the passage number for cells in SC increased, the length of time that cells had been maintained in EFP also increased. For determination of population doublings in primary cultures, it was assumed that each cell colony was derived from a single adherent hMSC (colony forming unit-fibroblastic [CFU-F]). We used an estimated average of 300 colonies for calculating cell proliferation. This estimated colony number is based on determination of colony numbers during approximately 20 years of experience with hMSCs. As described elsewhere [21], the number of population doublings for primary cultures was determined by calculating the base 2 logarithm of the number of cells harvested per culture dish at the end of primary culture divided by the average number of colonies. For all other passages, the number of population doublings was determined by calculating the base 2 logarithm of the number of cells per dish divided by the number of cells seeded × 0.85 (the approximate plating efficiency for these cells). Cumulative populations for any time were determined by adding the number of doublings for all passages up to that time.

**MSC Differentiation**

After the cell numbers were determined at the end of first and successive passages, some of the cells in standard conditions were seeded as cells of the next passage. The remaining SC cells and all of the EFP cells that were collected after trypsinization were used in the differentiation conditions described below.

**Chondrogenic Differentiation and Analysis**

As described elsewhere [20, 21, 24–25], hMSCs were cued to differentiate along the chondrogenic lineage at the end of first and all later passages. After trypsinization, hMSCs were resuspended in serum-free medium, centrifuged, and then resuspended in a pellet base medium consisting of Dulbecco’s modified Eagle’s medium with high glucose (DMEM-HG) supplemented with 1% antibiotic-antimycotic solution, 1% sodium pyruvate, and 1% nonessential amino acids (all obtained from In Vitrogen); also included were 1% ITS (BD Bioproducts, Billerica, MA, http://www.bdbiosciences.com), 10⁻⁷ M dexamethasone (Sigma-Aldrich), and 80 μM ascorbic acid-2 phosphate (Wako Chemicals USA, Richmond, VA, http://www.wakousa.com). The cells were centrifuged and resuspended in pellet base medium, to which transforming growth factor (TGF)-β1 (10 ng/ml) had also been added, at a density of 1.25 million cells per milliliter. Two hundred microliters of this cell suspension (250,000 cells) was added per well of a 96-well polypropylene V-bottom, multiwell dish (Phenix Research, Candler, NC, http://www.phenixresearch.com). The multiwell plates were centrifuged at 500 g for 5 minutes and then incubated at 37°C. The culture medium (pellet base medium plus TGF-β1) was changed every other day. Pellets were harvested after 14 and 21 days for histologic and biochemical analysis.
For histologic analysis, replicate samples were fixed overnight with 10% phosphate-buffered formalin (PBF) (Fisher Scientific International, Hampton, NH, http://www.fisherscientific.com), rinsed with Tyrode’s salt solution, and then stored in 70% ethanol until they were embedded in paraffin. Paraffin-embedded samples were cut into sections 5 μm thick, which were deposed onto glass slides and then deparaffinized and stained with toluidine blue.

Additional samples (usually five replicates per group) were collected in 1.5-ml tubes and frozen at −70°C for quantification of DNA and glycosaminoglycan (GAG) content as previously described [20, 21, 25]; chondroitin sulfate content was used as a measure of the quantity of GAG in each pellet [26]. After all of the frozen pellets had been collected for each experiment, they were then thawed at room temperature and digested in 200 μl of papain buffer (25 μg/ml papain, 2 mM cysteine, 50 mM sodium phosphate [disodium, anhydrous], 2 mM EDTA, pH 6.5). The samples were first refrozen at −70°C and then thawed and digested at 65°C for 3 hours, with vortexing every hour. A volume of 400 μl of 0.1 N NaOH was added to each tube and, after 30 minutes at room temperature, 400 μl of a neutralizing buffer was also added (4 M NaCl, 100 mM Na2HPO4; the pH was adjusted to 7.2, and 1.0 N HCl was then added to give a final concentration of 0.1 N HCl).

For quantification of pellet DNA, a 100-μl aliquot of the digest was combined with an equal volume of bisbenzimidide (1 μg/ml; Sigma-Aldrich) in each of four wells of a 96-well plate, and the absorbance was then read on a GENios Pro multiplate reader (Tecan, Durham, NC, http://www.tecan.com) with excitation and emission wavelengths of 340 and 465 nm, respectively. Concentrations of DNA were extrapolated from a standard curve generated with known concentrations of calf thymus DNA.

For quantification of GAG content, a volume of 25 μl of the pellet digest was combined with 250 μl of Safranin O (0.02% [wt/vol] in 50 mM sodium acetate, pH 4.8) per well of a 96-well dot-blot vacuum apparatus (both reagents from Sigma-Aldrich). A vacuum was applied to the apparatus, and the precipitates were collected on a nitrocellulose membrane; individual dots were cut from the membrane, transferred to 1.5-ml microcentrifuge tubes, and eluted with 10% cetylpyridinium chloride (Sigma-Aldrich) for 20 minutes at 37°C. Aliquots of the eluent were transferred to 96-well multiwell plates, and the absorbance was read at 536 nm. A standard curve was generated with known concentrations of chondroitin sulfate. GAG content determined for each pellet was normalized to the DNA content for the same pellet.

Adipogenic Differentiation and Analysis

Dissociation of hMSCs to adipocytes was induced by a modification [17] of the method described by Pittenger et al. [16]. hMSCs were resuspended in serum-free medium after trypsinization, and a portion of the cells was then centrifuged, resuspended in serum-containing DMEM-LG, and seeded into 35-mm dishes at 20,000 cells per cm². After 24 hours, medium was changed for all of the cultures. Adipogenic induction medium (AIM) was started on half of the cultures, whereas the other half received adipogenic maintenance medium (AMM, or control medium). Both AIM and AMM use DMEM-HG augmented with 10% FBS as a base medium. AMM also contains 10 μg/ml insulin, whereas AIM includes 100 μM indomethacin, 500 μM isobutylmethionxanthine, and 10⁻⁶ M dexamethasone in addition to insulin (all reagents from Sigma-Aldrich). Media were changed twice a week; after 10 days, cells in AIM were switched to AMM for the duration of their time in culture. After 21 days, cultures were fixed with 10% PBF for 1 hour, rinsed with 60% isopropyl alcohol, and then allowed to dry. When all of the experimental cultures were fixed, they were stained with Oil Red O, 3.5 mg/ml in isopropyl alcohol, diluted 3:2 with deionized water, for 10 minutes. Excess stain was removed, and the cultures were then rinsed four times with water and allowed to dry. Stain was eluted from the cells with 100% isopropyl alcohol for 10 minutes. The absorbance of the eluted stain was read at 492 nm; known concentrations of Oil Red O in 100% isopropyl alcohol were used as a standard curve [27].

In Vivo Osteogenic Induction and Analysis

First passage and later passage hMSCs were tested for their osteogenic potential in an in vivo assay in which the cells were loaded into porous ceramic cubes and implanted subcutaneously into immunocompromised mice. In this assay, which has been described previously [22, 28], cubes measuring 3 mm per side were cut from a cylindrical ceramic rod composed of 60% hydroxyapatite and 40% tricalcium phosphate generously provided by the Zimmer Corporation (Warsaw, IN, http://www.zimmer.com). The cubes were washed with deionized water, dried, and then autoclaved. The sterile cubes were immersed in a 100 μg/ml solution of fibronectin (BD Biosciences, Bedford, MA, http://www.bdbiosciences.com) in a 12 × 75-mm tube. A partial vacuum was produced by withdrawing air through the cap of the tube with a 30-ml syringe attached to a 22-gauge needle; the cubes were maintained in the fibronectin solution for 2 hours and were then transferred to a sterile 100-mm dish and allowed to dry overnight at room temperature inside a biological safety cabinet.

Cells from both standard and EFP cultures were trypsinized, resuspended in serum-free DMEM-LG, and counted; some of the cells were then resuspended in the same type of medium at a concentration of 5 × 10⁶ cells per milliliter in a 12 × 75-mm tube. Fibronectin-coated cubes were added to the cell suspensions, and a partial vacuum was produced as for fibronectin coating of the cubes. The cell-loaded cubes were incubated at 37°C for 2–4 hours and were then implanted subcutaneously in non-obese diabetic-severe combined immunodeficient mice that had been anesthetized with a rodent cocktail composed of ketamine, xylazine, and acepromazine (all obtained from Butler Schein Animal Health, Dublin, OH, http://www.butterschein.com) as described elsewhere [22]. The skin and subcutaneous tissue were anesthetized with 0.025% Marcaine (Butler Schein); a small incision was made on the dorsal surface of each mouse, and six to eight subcutaneous pockets were prepared via the incision with blunt dissection. One cube was placed in each pocket, and the incision was then closed with wound clips. After 6 weeks, the mice were sacrificed with an intraperitoneal injection of sodium pentobarbital, 0.01 ml per gram of body weight (Euthasol; Virbac Animal Health, Fort Worth, TX, http://www.virbacvet.com). All animal experiments were conducted in accordance with Case Western Reserve University School of Medicine protocol 2009-0138 and conformed to relevant regulations.

Histologic Processing

After the mice were sacrificed, the cubes were removed and immediately fixed with 10% PBF. Following fixation, the cubes...
were treated with a rapid decalcifying solution (RDO; Apex Engineering, Aurora, IL, http://www.rdodecal.com) and were then processed and embedded in paraffin with a Shandon automatic processor (Thermo Shandon Inc., Pittsburgh, http://www.thermo.com). Sections 5 μm in thickness were cut and deposited onto glass slides; the slides were then stained with Mallory-Heidenhain stain, coverslipped, and examined by bright-field microscopy for the presence of bone or cartilage. Individual sections were evaluated in a semiquantitative manner as described earlier [28–30]. Briefly, each section was given a score of between 0 and 4 based on the abundance of bone or cartilage. Scores of 1, 2, 3, and 4 indicate that up to 25%, 50%, 75%, or 100% of the pores, respectively, of an individual section of a ceramic cube contained bone or cartilage. An overall score for a cube was determined by calculating the average score for all sections. Finally, average scores and SDs were calculated for replicate cubes for each culture condition in each set of implants and for all implants.

Description of the Statistical Approach

The pellet culture data (DNA, GAG content, and GAG per DNA) used to generate Figure 1 were based on one representative experiment of four individual experiments, each involving cells from a different donor, in which chondrogenic differentiation was tested. Likewise, the data represented in Figure 2 were derived from one of four experiments that evaluated adipogenic differentiation. In each case, the data are representative of all experiments. (The data illustrated in Figs. 1 and 2 were derived from different experiments.) Because Figures 1 and 2 are based on single-donor preparations, these two data sets were analyzed separately using two-way analysis of variance followed by pairwise comparisons of standard and EFP groups at each passage. A similar approach was used to analyze for population doublings of human MSCs except that the ANOVA analysis also adjusted for (blocked on) sample preparation, since data were collected from several preparations. Data from in vivo osteogenesis experiments were also obtained from several preparations. To control for effects of preparation and because of non-normality of the data, these data were analyzed using the nonparametric van Elteren test, stratifying on sample preparation. Separate analyses were run to compare standard and EFP groups for passages 2–7. In all analyses involving comparisons of standard and EFP groups at multiple passages, a Bonferroni adjustment for multiple testing was used with an overall significance level of .05. That is, comparisons were significant at the .05 level only if the unadjusted p value was less than .05 divided by the number of passages examined.

RESULTS

Cell Morphology

Four to 6 days after the nucleated marrow-derived cells were seeded as primary isolates, a number of spindle-shaped and compact polygonal cells could be seen by phase-contrast microscopy. The adherent cells, or hMSCs, were, for the most part, organized into loosely structured colonies. Over the next week, the cells proliferated and the colonies became larger. Following subculture, the cells were no longer arranged into colonies but were instead distributed evenly over the culture substrate. As in the case of primary cultures, most of the cells appeared spindle-shaped, but some assumed a more compact triangular form or a broader, polygonal appearance. The top panel of Figure 3 depicts hMSCs on day 2 of the second passage.

Cells on day 7 of the third passage in SC featured some vacuoles and granules as cytoplasmic inclusions, and by day 7 of fourth passage the hMSCs were more varied in morphology. Some of the cells included thin, elongated processes, whereas others became wider and more polygonal. The majority of hMSCs, however, continued to exhibit a spindle-shaped form. Examples of these variations in the morphology of the cells are illustrated in the middle panel of Figure 3, which shows hMSCs on day 2 of the seventh passage.
Cells in extended first passage became confluent around day 7 or 8 and appeared to be multilayered by day 14. By day 20 of EFP, hMSCs had accumulated a number of vacuoles and granular inclusions. By day 27, the nuclei appeared more prominent than at earlier times, and the cultures were multilayered and dense. Dense cultures of hMSCs on day 44 of the first passage are shown in the bottom panel of Figure 3.

Cell Proliferation

The data illustrated in Figure 4 represent the average number of population doublings for five separate preparations of hMSCs maintained as standard or extended first passage cultures; however, the cells were cultured through seven passages for only three of the preparations and through six passages for four of the preparations. The cells proliferated most rapidly in primary culture, beginning with approximately 300 adherent cells per dish at the time of cell seeding (based on the estimated number of CFU-F) and progressing to an average of 3.11 million cells per dish on day 14. This represents a total of 12.5 population doublings. Population doubling data for the SC and EFP groups were identical at the end of primary culture and of first passage culture, since the cultures were not separated into two treatment groups until day 7 of first passage (day 21 of culture). By day 21 of culture, the number of cumulative population doublings was 15 for both groups. After day 21, the cells that were in SC proliferated more rapidly than those that had been maintained in EFP. Thus, by day 67 of culture, hMSCs in SC (day 8 of passage 7) had experienced 28.1 cumulative population doublings, whereas the number of cumulative population doublings for EFP cells was 16.9 (day 53 of passage 1). The differences in the number of population doublings were statistically significant ($p < .05$) for passages 2 through 7 and for comparable days of EFP. Clearly, maintaining hMSCs without subculturing them on a regular schedule resulted in a decrease in cell division.

Chondrogenic Differentiation

When hMSCs were cultured in chondrogenic pellet conditions as described in Materials and Methods, the cells formed a small sphere after 1 day in culture. After approximately 14 days, many of the cells had differentiated into chondrocytes, as indicated by their round configuration and the development of an extracellular matrix that stained metachromatically with toluidine blue. After 21 days in culture, both the number of chondrocytes and the amount of extracellular matrix increased. The former non-quantitative observation is based on examination of histologic sections by bright-field microscopy, whereas the latter quantitative finding is based on the difference in GAG content between pellets harvested on day 14 and those collected on day 21. Although there was some variation from one cell preparation to another, there was usually a 50%–60% increase in pellet GAG content from day 14 to day 21 when the data from both SC and EFP cultures at all passages were considered (SD, ±20; data not shown).
Solchaga et al. [20] have reported that the DNA content of pellet cultures does not vary significantly from day 7 to day 21 of a given passage and that GAG content of the pellets is maximal on day 21 of culture. Because of these observations, and for purposes of clarity, DNA and GAG content are shown here only for samples collected on day 21 for each passage.

Figure 1A depicts average DNA content for pellets prepared from cells in SC at the end of first through seventh passages and at comparable times for cells in EFP for a representative hMSC preparation. Data represent the average and SD for five pellets for each group. Since extended first passage did not begin until after day 7 of first passage, DNA values shown for the end of first passage apply to both groups. For other passages, DNA values were higher for pellets formed from cells cultured in EFP. The differences in DNA values between pellets formed from cells cultured in SC and those in EFP illustrated in Figure 1A were statistically significant at the end of passages 3, 5, and 7 with a confidence level of 95%. Through the period examined, DNA values for pellets containing hMSCs cultured in SC decreased slightly from the first to the seventh passage, except for a slight increase from passage 5 to passage 6. DNA content for pellets from cells in EFP also decreased with increasing passage number, but the decrease was neither as great nor as consistent as for DNA from cells in SC.

These data are representative of the other preparations of hMSCs analyzed with respect to differences between DNA content in SC and EFP. In fact, with a single exception, DNA values were higher for pellets formed from hMSCs in EFP than for those in SC at every passage of the four cell preparations examined (data not shown).

Likewise, again with a single exception, for the four individual donor-derived cell preparations analyzed, GAG content, expressed as micrograms of chondroitin sulfate per pellet, was higher at each passage for pellets constituted from cells cultured in EFP than for those formed from cells in SC. That exception (passage 3) can be seen in Figure 1B, which depicts GAG content for a typical hMSC preparation. The differences in GAG content between pellets initiated at the end of passages 2, 5, 6, and 7 of standard culture and the analogous pellets formed from cells at days 13 through 53 of extended first passage were statistically significant (\( p < .05 \)). As in the case of DNA content, there was a gradual decrease in GAG content with increasing passage number for both SC- and EFP-derived pellets.

The trend for GAG content normalized per microgram of DNA with increasing passage number was somewhat less consistent, as there was some variability from one passage to the next (Fig. 1C). However, except for pellets generated at the end of the third passage, GAG per DNA values for pellets formed from hMSCs in EFP were higher at each passage than for those formed from cells in SC. Most likely, the atypically low GAG values for the pellets formed between passages initiated at the end of passages 2, 5, 6, and 7 of standard culture and the analogous pellets formed from cells at days 13 through 53 of extended first passage were statistically significant (\( p < .05 \)). As in the case of DNA content, there was a gradual decrease in GAG content with increasing passage number for both SC- and EFP-derived pellets.

Histologic sections of representative pellets from passages 2, 4, and 6 of SC and from concurrent EFP cultures are shown in Figure 5. It should be noted that these pellets were from a different cell preparation than the one represented by the data in Figure 1; however, the main observations, namely, that pellets formed from cells in EFP were larger and had more abundant metachromatic extracellular matrix than those from cells in SC, were the same for all preparations examined. These pellets were...
fixed on day 21 of culture and stained with toluidine blue. In each pellet, the majority of hMSCs differentiated into chondrocytes, which appeared as large round cells surrounded by abundant matrix that stained metachromatically with toluidine blue. In some samples, cells in the central part of the pellets did not appear to be chondrocytes and lacked abundant matrix. This may have been due to decreased nutrient or oxygen accessibility in these areas of the pellets in the static culture medium of the 96-well dishes. Additionally, the cells at the periphery of the pellets were flattened and appeared fibroblast-like, as is typical for these preparations. It is noteworthy that, for each passage, the pellets formed from extended first passage cells were the same size or larger than those generated from standard cultures. This finding is consistent with the results of the quantification of GAG content (Fig. 1B) and with the observation that pellets from EFP cultures appeared to be larger, when viewed macroscopically, than their counterpart pellets from standard conditions (data not shown).

**Adipogenic Differentiation**

Human MSCs, whether cultured in standard conditions or in extended first passage, were capable of differentiating into adipocytes through seven passages when cultured in AIM. Cells maintained in AIM developed small lipid droplets beginning around 5 days of culture. The droplets increased in size and number through 21 days of culture; there were typically 20–50 droplets per differentiated adipocyte. Additionally, the cells assumed a more round to polygonal morphology, and over time, additional cells accumulated lipid (Fig. 2A). All cells in AMM, on the other hand, remained fibroblast-like in appearance, and lipid accumulation was not observed (data not shown).

The extent of differentiation for cells cultured in AIM, as indicated by a visual estimate of the amount of Oil Red O staining, decreased with increasing passage number and was higher at each passage for hMSCs in SC than for those at a comparable time in EFP. This visual observation was confirmed by spectrophotometric analysis of Oil Red O eluted from stained cultures (Fig. 2B). With the exception of the lower levels corresponding to second passage of cells in SC, the average amount of Oil Red O incorporated per culture decreased slightly with each passage, and the amount of stain was lower at each fixation time for cells in EFP than for those in SC. The differences between the SC and EFP cells were significant at passages 2, 4, 5, and 6 ($p < .05$).
Staining of hMSCs that had been cultured in AMM was minimal for both SC and EFP cultures, with the amount of eluted stain averaging less than 5% of that for cells in AIM (data not shown).

In Vivo Osteogenesis

Figure 6 shows histologic sections of two hMSC-loaded ceramic cubes stained with Mallory-Heidenhain stain. Cubes were loaded with hMSCs cultured in extended first passage (EFP) for 13 days (A) or 41 days (B). The majority of the pores of in both cubes contained bone, which had been deposited by osteoblasts (wide black arrows). Osteocytes, surrounded by osseous matrix, are indicated by white arrowheads. The qualitative morphological results illustrated here are typical of cubes loaded with EFP at other time points and with standard condition cells at any passage. Abbreviations: b, bone; BV, blood vessels; C, demineralized ceramic; F, fibrous tissue.

Staining of hMSCs that had been cultured in AMM was minimal for both SC and EFP cultures, with the amount of eluted stain averaging less than 5% of that for cells in AIM (data not shown).

In Vivo Osteogenesis

Figure 6 shows histologic sections of two hMSC-loaded ceramic cubes that were harvested after 6 weeks in vivo and processed as described in Materials and Methods. The cells in the cube shown in Figure 6A had been cultured in EFP for 13 days before implantation, whereas the cube shown in Figure 6B was loaded with cells cultured in EFP for 41 days. However, it should be noted that there were no qualitative differences in bone morphology among cubes loaded with hMSCs regardless of the culture conditions or length of time in culture. In our experience, some bone formation took place in nearly all of the hMSC-loaded ceramic cubes that were implanted into immunocompromised mice. Early bone development could be seen in cubes harvested after 3 weeks in vivo [14, 28]. At that early time point, a narrow band of bone that had been deposited by osteoblasts along the walls of individual pores within the cubes could be identified; an example of aligned osteoblasts after 6 weeks in vivo can be seen in both panels of Figure 6. By this later harvest time, the amount of bone had increased, and some fibrous tissue and vasculature could be seen on the inside aspects of the pores (Fig. 6). In fact, bone formation in these cubes was always associated with host vasculature. Some pores included only fibrous and vascular tissue and not bone. Cartilage was almost never observed in implanted cubes loaded with hMSCs.

A graphic comparison of the extent of bone formation by hMSCs in SC and EFP in this in vivo assay can be seen in Figure 7, which depicts the average scores and SDs for all cubes from three individual hMSC preparations. Bone formation in implanted ceramic cubes loaded with hMSCs cultured in SC declined after second passage, and by seventh passage these cells produced very little bone in this in vivo assay. On the other hand, the amount of bone in cubes loaded with hMSCs cultured in EFP remained relatively consistent through seven passages. Moreover, the cube scores for cells in EFP were consistently higher than for those containing cells cultured in the standard manner; the differences were statistically significant for cubes implanted at the end of passages 4 – 7 (p < .05).

DISCUSSION

Because the rate of proliferation for cells in culture usually decreases once they become confluent, cells are normally subcultured just before they reach this point. For some cells, simply increasing the frequency of changes of culture medium keeps the cells proliferating rapidly [31]. For other cells, especially immortalized cell lines and transformed cells, subculturing may be continued through a large number of passages without affecting the phenotypic characteristics of the cells. However, primary isolates of mammalian and avian cells, including osteoblasts and chondrocytes, lose their characteristic expression markers after extensive subculturing. Solchaga et al. reported that hMSCs show diminished chondrogenic potential in aggregate cultures after as few as four passages.
In addition to the diminished differentiation ability of the hMSCs with extensive subculturing, there has also been a concern that allowing the cells to become confluent may cause them to lose their stem cell potential. However, the results described here show that hMSCs retain their ability to differentiate into bone, cartilage, and fat even when the cells have been kept in first passage for up to 53 days and have become multilayered and confluent. In fact, the ability of hMSCs in extended first passage to differentiate along osteogenic and chondrogenic pathways was at least comparable to that of sister cells subcultured at the same time according to our standard protocols. Whereas the potential for adipogenic differentiation for the cells was significantly diminished relative to those in standard conditions by maintaining them as EFP cultures, the cells in EFP still accumulated substantial amounts of lipid (as seen in Oil Red O staining), whereas control cells for either group (those cultured with AMM) did not. Nevertheless, Oil Red O staining was consistently higher for cells in SC than for those in EFP, and the differences were significant at several time points, indicating that differentiation to adipocytes was greater in SC than in EFP. On the other hand, consistently and (for some passages) significantly higher pellet GAG values and ceramic cube scores for hMSCs cultured in EFP suggest that differentiation into cartilage and bone was greater for hMSCs in those conditions.

High-density conditions are required for the development of cartilage in pellet cultures and in stage 24 chick embryonic limb bud cultures [5], and it is possible that maintaining hMSCs in very dense conditions in EFP predisposes the cells toward chondrogenesis and enhances their differentiation into this lineage. Moreover, repeated trypsinization of hMSCs in SC might have an adverse affect on the ability of the cells in standard conditions to differentiate along the osteogenic and chondrogenic pathways.

Because individual cells are difficult to visualize once the EFP cultures become confluent, it is difficult to compare late passage morphological features for hMSCs in the two conditions. Thus, whether the EFP cells develop some of the same characteristics noted for cells in standard conditions in late passages (i.e., that they are more spread and feature longer processes) is not clear.

In contrast to the effects of extended first passage on differentiation, the proliferation of hMSCs in EFP was greatly diminished relative to that of cells in SC. Whereas the latter attained more than 27 cumulative population doublings after 60 total days of culture, cells in EFP reached only 17 doublings. This is an important consideration for potential tissue engineering or regenerative medicine applications, since culture expansion may be critical to obtaining adequate cell numbers for these applications. Thus, maintaining cells for extended periods in first passage is not recommended as a standard method of hMSC culture.

Rather, the information adds to our understanding of the nature of these cells and allows more flexibility in the timing of subculturing and the use of the cells. For example, cultures of hMSCs may be approaching confluence in anticipation of their use with cultures of other cells or in an animal model. A delay in the proliferation of the other cells or some problem with the readiness or availability of animals may complicate the timing of trypsinization of the hMSCs. The results presented here indicate that such a delay need not compromise the stem nature of the cells and thus may not necessarily affect the experimental outcome.

It should also be noted that hMSC cultures maintained in the first passage for more than 50 days were not more difficult to release from the culture substrate with trypsin than were those in standard conditions at a comparable time. This contrasts with hMSCs in osteogenic conditions (i.e., dexamethasone-containing medium), which, in our experience, become very trypsin-resistant after only 8 days in culture.

Finally, it should be noted that although comparisons are drawn in this report between SC and EFP cultures at comparable times in terms of days in culture, hMSCs in standard conditions had undergone more population doublings than had those in EFP at any time after day 7 of first passage (Fig. 4); thus, cells in SC may be considered “older” in terms of population doublings, although they are the same age in terms of calendar days. In other words, a consideration of the number of population doublings may represent a more valid indicator of the age of hMSCs in the two conditions described here than does the chronological age of the cultures. Nevertheless, we consider the ability of hMSCs to retain their differentiation potential under superconfluent conditions late into the first passage to be an unexpected outcome for these experiments.

CONCLUSION

First passage hMSCs were able to differentiate into fat, cartilage, and bone even after having been maintained for long periods of time (up to 53 days) without being subcultured, although the culture medium was changed twice each week. In this regard, they were similar to hMSCs cultured for the same length of time but subcultured on a weekly basis. However, the rate of proliferation of cells in standard conditions was significantly higher than that of cells in extended first passage. Since very large numbers of cells will certainly be needed for some translational medicine therapies, subculturing hMSCs on a regular basis should remain the standard procedure for propagating these cells.

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AUTHOR CONTRIBUTIONS

D.P.L.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; M.D.S.: data analysis and interpretation, manuscript writing; A.I.C.: financial support, administrative support, provision of study materials, data analysis and interpretation, manuscript writing, final approval.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.
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