ORIGINAL ARTICLE

Human and Rat Bone Marrow-Derived Mesenchymal Stem Cells Differ in Their Response to Fibroblast Growth Factor and Platelet-Derived Growth Factor

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Tissue engineering requires large numbers of cells with enhanced differentiation properties. Thus, the effect of expansion conditions must be explored. Human and rat marrow-derived mesenchymal stem cells (hMSCs and rMSCs, respectively) were comparatively culture expanded through seven passages in the presence of either fibroblast growth factor-2 (FGF-2) or platelet-derived growth factor BB (PDGF-BB). Proliferation of both hMSCs and rMSCs was enhanced by FGF-2 and PDGF-BB. Population doubling times for hMSCs were 2.4 days for control and 1.75 and 2.0 days for FGF-2 and PDGF-BB, respectively, and 3.25, 3.06, and 2.95 days for rMSCs. Supplementation with FGF-2 during cell expansion resulted in significantly greater *in vivo* bone formation for hMSCs. Use of PDGF-BB resulted in greater bone formation than that observed for control conditions, but the differences were only significant for P1. For rMSCs, significant increases in bone formation were noted in either FGF-2 or PDGF-BB expanded cells implanted at P4 or P7, but not for P1. Under *in vitro* osteogenic stimulation, calcium content was elevated and bone matrix deposition was enhanced for P1 and P7 rMSCs expanded with FGF-2. Although culture conditions, including FBS, were held constant, these observations suggest that medium must be optimized separately for each species of MSCs.

Keywords: mesenchymal stem cells, PDGF-BB, FGF-2

Impact Statement

The presented research emphasizes the importance of coordinated approaches in Tissue Engineering and cell culture involving growth factor supplementation of growth medium that involves complex effects on cells; these effects are species-specific and are understudied. Therefore, the importance of medium optimization for MSCs from different species. The effect of PDGF-BB on bone-marrow derived MSC differentiation and proliferation itself is understudied and under-reported.

Introduction

TISSUE ENGINEERING CONCEPTS include three central components as follows: cells, growth factors to modulate proliferation or function of cells, and some type of scaffold to which the cells can attach and which, in turn, physically supports the cells and facilitates their proliferation, differentiation, and eventual engraftment into the host tissue. Mesenchymal stem cells (MSCs) have frequently been cited as potential candidates for tissue engineering applications, particularly for cartilage and bone.^{1–4}

Maximizing the proliferative potential of these MSCs and optimizing their potential to differentiate along se-

lected pathways are both critical to their use in such applications. Several methods are available to achieve these ends, including the use of cytokines and growth factors such as fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and platelet-derived growth factor (PDGF).^{5–7}

In our laboratory, Hanada *et al.*⁸ demonstrated that FGF-2 and BMP-2 increased proliferation on both *in vitro* and *in vivo* osteogenesis of rat MSCs (rMSCs). In this study, rMSCs were exposed to one or both of the cytokines for a brief (6-day) period in first passage (P1). The present study, by comparison, examines FGF-2 and PDGF-BB in rMSC cultures, but extends the period under investigation

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through seven passages. Stimulation of mitogenesis and preservation of chondrogenesis in human MSCs (hMSCs) cultures expanded in the presence of FGF-2 were described by Refs.^{9,10}

Materials and Methods

Culture of hMSCs

Human MSCs were isolated as previously described.^{11–15} Briefly, marrow was collected by needle aspiration from the posterior-superior iliac crest of healthy adult donors ($n=11$). The marrow was collected using a procedure reviewed and approved by the University Hospitals of Cleveland Institutional Review Board; informed consent was obtained from all deidentified donors. Approximately 20 mL was collected from each donor in a syringe containing 2 mL of heparin (400 units/mL). The marrow was centrifuged for 15 min at 480 g on a preformed Percoll gradient (density 1.03–1.12 g/mL; Sigma, St. Louis, MO). The nucleated cells of the monocyte light-cell fraction were collected, rinsed with serum-containing Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Invitrogen, Grand Island, NY), centrifuged at 450 g , resuspended, counted, and seeded in standard tissue culture vessels at 1.8×10^5 cells per centimeter.^{2,15}

Culture medium was changed after 4 days and every 3–4 days thereafter. At the time of the first change of medium, cells were either continued in DMEM-LG supplemented only with selected lots of 10% FBS (Invitrogen) (control conditions)¹⁵ or were switched to medium further augmented with basic FGF-2 or PDGF, both at 10 ng/mL. Cells were continued in their respective medium throughout their time in culture with complete replacement every 2–3 days.

hMSCs were continued in primary culture until colonies became large but were not multilayered. Normally, primary hMSC cultures are trypsinized after 14 days; however, cultures maintained in the presence of the growth factors used here required trypsinization after 10–12 days. To keep cultures in each of the conditions at the same passage and day of culture, all groups were trypsinized at the same time. The result of this practice was that colonies in control cultures were usually smaller than those exposed to FGF-2 or PDGF. The cells were seeded at 4500 cells per cm^2 and continued in culture as the next passage. Medium was changed as for primary culture. Cells were maintained in culture through 7th passage, and in some cases, through 10th passage, always in the same medium conditions to which they had been introduced on day 4 of primary culture.

Culture of rMSCs

Cultures of rMSCs were established as previously described.^{16–18} Femora and tibiae were aseptically removed from three F-344 rats, 2–6 months old, after the animals had been euthanized by CO_2 narcosis in accordance with an IACUC approved protocol. Marrow was flushed from the long bones and mechanically disrupted by passage through successively smaller-gauge needles. The marrow was rinsed in α -MEM supplemented with 10% FBS (Gemini Bio Products, West Sacramento, CA) from a lot selected for optimal *in vitro* proliferation and for *in vitro* and *in vivo* osteogenic differentiation of rat MSCs.¹⁹ The nucleated cells were

centrifuged, resuspended in serum-supplemented medium, counted, and seeded at 9×10^5 cells per cm^2 .

Medium changes, addition of growth factors, where applicable, and methods and timing of cell trypsinization were essentially the same as for hMSCs, except that subcultured rMSCs were seeded at 12,500 cells per cm^2 .

In vivo osteogenesis

At the end of first, fourth, and seventh passage, some of the cells harvested during trypsinization were used for an *in vivo* assay for osteogenesis. The methodology for this assay has been described previously.^{14,17} Briefly, cubes measuring ~ 3 mm per side were cut from a rod of porous ceramic composed of 60% hydroxyapatite and 40% tricalcium phosphate generously provided by the Zimmer Corporation (Warsaw, IN). The cubes were washed with deionized water, dried, and then autoclaved. The sterile cubes were immersed in a solution of human fibronectin (Corning, Bedford, MA) at a concentration of 100 $\mu\text{g/mL}$ in Tyrode's salt solution. After 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 kept in the fibronectin solution for 2 h, after which they were allowed to dry at room temperature.

Rat or human MSCs that had been cultured with or without growth factors were resuspended in medium without serum or growth factors at a concentration of 5 million cells per milliliter. Fibronectin-coated cubes were added to the cell suspensions, and a partial vacuum was generated to infiltrate the pores of the cubes with the cell suspension. Cell-loaded cubes were then incubated at 37°C for 2 h in a humidified atmosphere of 5% CO_2 and 95% air. After the incubation period, individual cubes were implanted subcutaneously on the dorsal surface of either severe combined immunodeficient mice (for hMSCs) or syngeneic rats (for rMSCs).

Rats and mice were anesthetized with a rodent cocktail consisting of ketamine, xylazine, and acepromazine as described elsewhere.¹⁴ The skin and subcutaneous tissue at the incision site were injected with 0.1 mL of the local anesthetic Marcaine at a concentration of 0.25% for rats or 0.025% for mice. After the incision was made on the dorsal surface, up to nine subcutaneous pockets were expanded by blunt dissection. One cube was placed in each pocket, and the incision was then closed with wound clips. Animals were euthanized after 6 weeks, and the cubes were fixed with 10% phosphate-buffered formalin.

Histologic processing and analysis

After fixation, the ceramic cubes were decalcified with RDO (Apex Engineering, Aurora, IL) and processed and embedded in paraffin with a Shandon automatic processor. Sections 5 μm in thickness were cut and stained with toluidine blue or Mallory Heidenhain, coverslipped, and examined by brightfield microscopy for the presence of bone or cartilage. Individual sections were evaluated in a semi-quantitative manner as described earlier.²⁰ Briefly, each section was given a score of between 0 and 4 based on the abundance of bone and cartilage. Scores of 1, 2, 3, and 4 indicated, respectively, that up to 25%, 50%, 75%, or 100% of the pores in an individual section of a ceramic cube contained bone or cartilage. The overall score for a cube was determined by calculating the average score for all sections;

the average score has been shown to correlate with a histomorphometric analysis.²⁰ Finally, average scores and standard deviations were calculated for replicate cubes for each medium condition in each set of implants.

Population dynamics

MSCs for each medium condition were counted by hemacytometer at the end of each passage. Determination of population doublings in primary cultures assumes that each cell colony (colony forming unit-fibroblastic [CFU-F]) is derived from a single adherent fibroblast-like cell (MSC).²¹ In some cases, the number of CFU-F was determined by counting colonies in cultures fixed with 10% phosphate-buffered formalin for 15 min and then rinsed with Tyrode's salt solution and stained with 0.1% crystal violet for 30 min. Where the number of cultures was insufficient to permit determination of actual CFU-F, an estimated average colony number was used. These numbers (300 for hMSCs and 500 for rMSCs) are based on typical colony numbers determined over ~27 years of experience with these cultures.

The number of population doublings for primary cultures was determined by calculating the base 2 log 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 log of the number of cells per dish divided by the seeding density $\times 0.85$ (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.

In vitro osteogenesis

Second passage cultures of rMSCs were seeded into 35-mm dishes at 10^5 cells per cm^2 . On day 1, the day following seeding, culture medium was changed on all dishes. Half of the cultures received medium containing osteogenic supplements (OSs), consisting of DMEM-LG augmented with 10% FBS, 80 μM ascorbic acid 2-phosphate, and 10^{-7} M dexamethasone. The remaining dishes received control medium, which is the same as OS medium, but without dexamethasone. Beginning on day 10, the media for both OS and control cultures were also supplemented with 2 mM β -glycerophosphate. Both OS and control media were changed twice weekly.

von Kossa staining

The presence of mineralized deposits in second passage rMSC cultures was demonstrated by von Kossa staining. On day 21, triplicate cultures of OS and control cultures were rinsed twice with Tyrode's salt solution, fixed with 10% phosphate-buffered formalin for 15 min, and rinsed thrice with distilled water. One milliliter of 2% (w/v) silver nitrate (Sigma Chemical) was added per dish, and the cultures were placed in a dark environment for 10 min; cultures were then rinsed thrice with distilled water and exposed to bright light (while covered with water) for 15 min. Culture dishes were rinsed again with water and then dehydrated with 100% ethanol.

Calcium assay

On day 21, triplicate rMSC cultures were rinsed twice with Tyrode's salt solution and fixed with 10% phosphate-

buffered formalin for 30 min. Following fixation, cultures were rinsed twice with distilled water, allowed to dry, and stored at 4°C. Calcium was extracted with 1 mL of 0.6 M HCl per dish; dishes containing this solution were placed on a rotary shaker and maintained at a speed of 50 rpm overnight. Aliquots of the extract, diluted as needed for spectrophotometry, were added to wells of a 96-well culture dish (4 wells per sample); reagents from a Commercial Calcium Assay Kit (Biotron Diagnostics, Milpitas, CA) were added to the wells, and the absorbance was read at 575 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA). Calcium concentration was determined with a standard curve generated from a series of dilutions of CaCl_2 .

Statistical methods

Human MSC ceramic cube dataset contained data from experiments involving 9 donors and consisted of bone formation scores from 275 ceramic cubes obtained from a total of 50 mice. Data from P1, P4, and P7 comparing control and two growth factor treatments (control, FGF-2 [10 ng/mL], and PDGF [10 ng/mL]) are analyzed in this study. Hereafter, for hMSCs but not for rMSCs, the conditions will be referred to as control, FGF, and PDGF.

Rat MSC ceramic cube data consist of bone formation scores from implanted cubes, comparing four growth factor treatments (control, FGF-2, 1 ng/mL, FGF-2, 10 ng/mL, and PDGF, 10 ng/mL [written hereafter as control, FGF-1, FGF-10, or PDGF]) at three passages (1, 4, 7). The data consisted of cells from seven different preparations of rMSCs. A total of 314 ceramic cubes were harvested from a total of 54 syngeneic host rats.

For both hMSCs and rMSCs, a linear mixed repeated measures model was used to analyze and compare mean scores among treatments within each passage and among passages for each treatment. The model included random effects for donor and host mouse or rat. For hMSCs, pairwise comparisons of the three treatments at each passage, and of the three passages for each treatment, were made using a Bonferroni correction for multiple testing based on three comparisons at each passage or treatment.

For rMSCs, estimated means from the model comparisons control for the fact that not all treatments were studied for every donor/passage combination. Pairwise comparisons of the four treatment means at each of the three passages were made with the Bonferroni method, adjusting for six pairwise comparisons. Pairwise comparisons of means across the three passages for each treatment were made with a Bonferroni correction, adjusting for three pairwise comparisons. For both hMSCs and rMSCs, adjusted *p*-values are presented, where adjusted *p*-values lower than 0.05 are considered statistically significant.

Analyses were completed for calcium content determined for rMSCs cultured in the four growth factor treatment conditions described above, either with and without OS, at passages 2 and 7.

For each combination of passage (P2 or P7) and osteogenic supplement status (OS, no OS), calcium concentrations of the four treatment groups were analyzed using a one-way analysis of variance. The four treatments' group means were compared in pairwise manner using the Bonferroni correction for multiple testing, adjusting for six

pairwise comparisons. Student's *t*-tests assuming unequal variances were used to compare cultures receiving the same treatment, but differing in OS status, separately by passage (P2 or P7), using a Bonferroni adjustment for multiple testing with four comparisons. Adjusted *p*-values from the Bonferroni method are presented, adjusting for the four comparisons at each passage. In all analyses, adjusted *p*-values less than 0.05 are considered statistically significant after correction for multiple testing.

Results

Cell morphology

In primary cultures of both human and rat MSCs, individual adherent fibroblast-like cells, along with many non-adherent cells, can be seen after 2 or 3 days of culture. The nonadherent cells are removed during the process of changing culture medium over the course of primary culture, and the fibroblastic cells (MSCs) proliferate and form colonies. The majority of cells in the colonies are spindle shaped. The colonies increase in size as the cells proliferate and primary control cultures are usually subcultured after 14 days. Human MSCs treated with FGF are smaller in size^{9,10} than those cultured with FBS alone or with PDGF. Furthermore, because human and rat MSCs in FGF- and PDGF-containing medium proliferate more rapidly than cells in control medium, they are usually subcultured at 12 or 13 days of primary culture. In both cultures, it is clear that the attached cells are not homogeneous in their basic morphology, and, in primary culture, clearly some of the cells are of the monocyte/macrophage lineage. Subcultured hMSCs retain the spindle shape form seen in primary culture, and cells treated with FGF are smaller than the control cultures through repeated passages. Human MSCs in control medium, while maintaining their spindle shape, become larger and more spread in later passages (Fig. 1).

Some rMSCs are spindle shaped as for hMSCs, but many are broader and more polygonal in morphology. With repeated passaging, the rMSCs in control medium took on this polygonal and spread appearance, while more of the cells in FGF- or PDGF-containing medium remained spindle shaped. There was no apparent difference in size of rMSCs maintained in FGF or PDGF (Fig. 2).

Proliferation of human MSC

A concentration of 10 ng/mL has previously been shown to be optimal for both FGF and PDGF in promoting the proliferation and chondrogenic potential of hMSCs.¹⁰ The same concentration was used to evaluate the effect of these growth factors on cells grown continuously in their presence on osteogenic differentiation *in vivo*. Concentrations of 1 and 20 ng/mL were also evaluated in this study, but were not found to be superior to the previously established concentration of 10 ng/mL (data not shown). Figure 3 illustrates the average number of cumulative population doublings through seven passages for hMSCs in medium augmented with only 10% FBS and in medium also supplemented with FGF or PDGF at 10 ng/mL.

These results are derived from seven hMSC preparations through P4, six preparations each for P5 and P6, and three preparations for P7. For each preparation, population doublings were the shortest in FGF-supplemented medium and longest in control medium. On average, cells in each medium condition proliferated very rapidly from the time they were seeded as primary isolates to the end of primary culture (day 14). Thus, on day 14 of first passage, the number of cumulative population doublings was 11.5 for control cultures and 12.3 and 12.4 for hMSCs cultured in PDGF and FGF-2, respectively. Although the rate of cell proliferation decreased thereafter through time in culture, the decline in the rate of proliferation was less pronounced in cells cultured with growth factor supplementation. As a result, the

FIG. 1. Phase contrast photomicrographs of hMSCs cultured in control medium (on left) or in medium supplemented with FGF (center panel) or PDGF (on right) on day 2 of culture in P1 (top row) or P7 (lower row). Scale bars = 200 μm. FGF, fibroblast growth factor; hMSC, human mesenchymal stem cells; MSCs, mesenchymal stem cells; PDGF, platelet-derived growth factor. Color images available online at www.liebertpub.com/tea

FIG. 2. Phase contrast micrographs of rMSCs cultured in control medium (*left*) or in medium augmented with FGF-2 (1 ng/mL) (*center*) or PDGF (10 ng/mL) (*right*) on day 3 of P1 (*top row*) or day 1 of P7 (*lower row*). Scale bars = 200 μ m. rMSC, rat mesenchymal stem cells. Color images available online at www.liebertpub.com/tea

total number of population doublings by the end of P7 (59 days after cell isolation) was 25.4, 29.8, and 33.3 for cells in control medium, PDGF-containing medium, and FGF-containing medium, respectively.

Proliferation of rMSC

Rat MSCs were cultured in α -MEM supplemented with 10% FBS (control medium) or in the same medium also supplemented with FGF-1, FGF-10, or PDGF. The selection of these concentrations was based on previously published

studies.¹⁰ Although rMSC proliferation in FGF-10 was greater than for control medium and sometimes greater for PDGF at the same concentration, the morphology of the cells cultured in FGF-10 was atypical, especially for cells in later passages (P4 and later). Notably, the cell surface of FGF-10-supplemented rMSCs was very irregular, and cellular debris was present in the cultures. Moreover, β -galactosidase staining of the cells, indicative of cell senescence, was much higher than for cells in PDGF or in control medium (data not shown); cell staining for β -galactosidase was done with reagents from a commercially available kit

FIG. 3. Effect of FGF and PDGF on hMSC proliferation. Cumulative population doublings are depicted for a typical preparation of hMSCs cultured in control medium (*filled diamonds, solid line*), in medium supplemented with FGF-2 (*filled squares, short dashed line*), or with PDGF (*filled triangles, long dashed line*). Data points indicate the number of accumulated population doublings at the end of each passage from P0 (day 14) through P7 (day 58).

according to the manufacturer's instructions (Sigma Chemical, St. Louis, MO). As a result of these observations, a range of concentrations of both FGF and PDGF was tested for rMSCs. The optimal concentrations, based on cell morphology and proliferation, were determined to be 1 ng/mL for FGF and 10 ng/mL for PDGF.

Proliferation data for rMSCs are depicted in Figure 4, which illustrates cumulative population doublings through seven passages for a preparation of rMSCs in control medium and in medium further supplemented with growth factors. In this particular preparation, cells were expanded in medium supplemented with FGF-1 and FGF-10, or with human PDGF at 10 ng/mL, in addition to control medium. The data for FGF-10 are not shown, in part, because they are comparable to those for FGF-1 and also to make Figure 4 easier to visualize.

As in the case of human MSCs, cell proliferation was very rapid throughout primary culture; thus, at P1 (day 14) the number of cumulative population doublings ranged from 11.8 for control cultures to 13.1 for cells in PDGF-containing medium. These numbers are typical population doubling values for P1 cultures for all of the preparations tested. Cell proliferation continued through the 58 days that the cells were maintained *in vitro*, although, as was the case with hMSCs, the rate of division decreased over time for all of the conditions, as indicated by a lower number of population doublings, in general, at successive passages. In this preparation the highest number of population doublings was for PDGF (19.85). The number of doublings for FGF-1 was nearly the same as for FGF-10 (18.3 and 18.8, respectively). The number of population doublings for each of these conditions exceeded that for rMSCs in control medium (16.5). Throughout this article, concentrations of 1 and 10 ng/mL of FGF-2 shall be assumed to be the standard conditions for rat and human MSCs, respectively.

Comparison of human and rat MSC proliferation

Thus, for both human and rat MSCs ~12–13 population doublings occur between the seeding of the cells on day 0 and 14 of primary culture, when the cells are normally subcultured for the first time. However, after first passage, proliferation of hMSCs, as indicated by the number of accumulated population doublings, is faster than for rMSC. The average population doubling time for cells in control medium (the time from cell seeding through the end of passage 7 for six hMSC and eight rMSC preparations) was 2.4 days for hMSCs and 3.25 days for rMSCs. This equates to the average number of population doublings per day of 0.43 for hMSCs and 0.31 for rMSC. For hMSCs, supplementation of medium with 10 ng/mL FGF increases the number of population doublings per day from 0.43 to ~0.59, while augmentation with PDGF, also at 10 ng/mL, increases the number of doublings per day to 0.51. These data translate to increases of 37% and 19%, respectively, in the number of population doublings per day.

By comparison, the increase in proliferation for rMSCs with inclusion of these growth factors was more modest than for hMSCs. The use of FGF-1 increases the average number of population doublings per day from 0.31 to 0.33 (5.2% increase), and supplementation of the culture medium with PDGF changes this value to 0.34 per day (an increase of 9.4%). The use of FGF-10 resulted in the same number of population doublings per day as for PDGF, but as indicated earlier, this concentration resulted in undesired morphological changes and, as will be seen later, in decreased osteogenic differentiation.

Finally, the more rapid proliferation of hMSCs (relative to rMSCs) was reflected in the higher number of population doublings at end of P7 for each of the medium conditions. In control medium, for example, rMSCs attained 16.5 populations by day 58, while hMSCs reached 23.7 by day 59.

FIG. 4. Effect of FGF and PDGF on proliferation of rMSCs. Cumulative population doublings are shown for a typical preparation of rMSCs cultured in control medium (filled diamonds, solid line), in medium supplemented with FGF-2 at 1 ng/mL (filled squares, long dashed line), or in human (filled circles, short dashed line) or rat (open squares, dash-dot line) PDGF at 10 ng/mL. Data points indicate the number of accumulated population doublings at the end of each passage from P0 (day 14) through P7 (day 58). Population doublings for rMSCs in medium containing FGF-2 at 10 ng/mL are comparable to those for FGF-2 at 1 ng/mL (data not shown).

TABLE 1. HUMAN MESENCHYMAL STEM CELLS IN CERAMIC CUBES *IN VIVO*: SUMMARY STATISTICS FOR THE *IN VIVO* BONE SCORES ACCORDING TO TREATMENT AND PASSAGE

Passage	Condition	N	Mean	SD	Cell preps	Model-based mean	SE
1	Control	30	0.72	0.78	8	0.78	0.18
	FGF	28	1.70	0.82	8	1.73	0.19
	PDGF	29	0.99	0.96	8	1.07	0.18
4	Control	36	0.45	0.56	9	0.45	0.18
	FGF	35	1.17	0.68	9	1.19	0.18
	PDGF	36	0.56	0.59	9	0.59	0.18
7	Control	28	0.17	0.30	7	0.28	0.19
	FGF	30	0.68	0.49	7	0.82	0.19
	PDGF	23	0.24	0.34	6	0.46	0.19

FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; SD, standard deviation; SE, standard error.

The greatest number of population doublings at the end of P7 for rMSCs was seen with those cultured in PDGF (19.8), while proliferation of hMSCs was maximal when they were cultured in the presence of FGF (31.1 doublings). It is also noteworthy that the number of population doublings for all medium conditions for hMSC was greater than for any condition for rMSCs at the end of P7.

Ceramic cube assay

Human MSCs. The results of the *in vivo* ceramic cube assay for hMSCs are listed in Table 1 and depicted in Figure 5. The average ceramic cube scores are shown for hMSCs cultivated in control medium (C) or in medium supplemented with FGF (F) or PDGF (P), both at 10 ng/mL. Data are shown for cubes loaded with these cells implanted at the end of P1, P4, and P7. At each passage, the amount of bone present in the cubes loaded with cells cultured in the presence of FGF is significantly greater than for those loaded with cells cultured in control medium or in medium augmented with PDGF ($p < 0.0001$; Table 2). More bone was detected for cubes containing hMSCs cultured in PDGF-supplemented medium than for control hMSCs; while this

holds for each passage, a statistically significant difference exists only for P1 ($p < 0.02$). For each of the three conditions, the average amount of bone per cube decreases with increasing passage number. With the exception of the difference between P4 and P7 cube scores for control and PDGF cells, the differences among passages for each medium condition are significant ($p < 0.05$). The data illustrated in Figure 5, along with the number of cell preparations and individual cubes on which the data are based, are shown in Table 1.

Rat MSCs. The effect of FGF-2 and PDGF on *in vivo* osteogenesis for rMSCs is illustrated in Figure 6 and in Table 3. Several major differences can be seen when comparisons are made between the ability of human and rat MSCs cultured with or without growth factors to form bone in ceramic cubes *in vivo*. First, unlike the results for hMSCs, where cube scores for cells cultivated in the presence of FGF-10 were significantly higher than any of the other cell groups at each passage, cube scores for rMSC cultured with FGF-10 were significantly lower than for the other cell treatment groups at each passage; the differences were significant except for those between control and FGF-10 at P4 and P7 (Table 4). This finding is consistent with the atypical

FIG. 5. Effect of FGF-2 and human PDGF on *in vivo* bone formation for rMSC. Bars indicate the average histologic scores for cell-loaded cubes harvested 6 weeks after implantation; scores are based on the amount of bone found in cube pores. Human MSCs had been cultured in control medium (vertical lines), in medium supplemented with FGF-2 (open bars), or in medium with PDGF (diagonal lines). Both growth factors were at a concentration of 10 ng/mL. Error bars indicate standard deviation, and asterisks indicate that the scores for cubes with cells in FGF-2-containing medium were significantly higher than those in control or PDGF-containing medium ($p < 0.01$).

TABLE 2. HUMAN MESENCHYMAL STEM CELLS IN CERAMIC CUBES *IN VIVO*: STATISTICALLY SIGNIFICANT DIFFERENCES DETERMINED FOR CERAMIC CUBE SCORES IN DIFFERENT MEDIA CONDITIONS FOR CELLS IN PASSAGES 1, 4, AND 7

Passage	Comparison	Adjusted p-value	Condition	Passage comparison	Adjusted p-value
1	Control vs. FGF	<0.0001	Control	1 vs. 4	0.0499
	Control vs. PDGF	0.0176		1 vs. 7	0.0031
	FGF vs. PDGF	<0.0001		4 vs. 7	0.6547
4	Control vs. FGF	<0.0001	FGF	1 vs. 4	0.0004
	Control vs. PDGF	0.3972		1 vs. 7	<0.0001
	FGF vs. PDGF	<0.0001		4 vs. 7	0.0303
7	Control vs. FGF	<0.0001	PDGF	1 vs. 4	0.0020
	Control vs. PDGF	0.3674		1 vs. 7	0.0004
	FGF vs. PDGF	<0.0001		4 vs. 7	1.0000

Adjusted *p*-values were calculated using a Bonferroni correction for three pairwise comparisons. Adjusted *p*-values less than 0.05 are statistically significant at overall significance level (Family wise error rate) of 0.05.

in vitro rMSC morphology and increased cell senescence staining associated with this FGF-2 concentration. In contrast, significant increases in bone development in the cube model, relative to cubes loaded with control or FGF-10-cultured cells, were seen for rMSCs cultured with either FGF-1 or with PDGF for those cubes implanted at the end of P7 ($p < 0.0001$). For P4 cells, the mean cube score for PDGF-cultured cells was significantly higher than for cubes loaded with control cells ($p = 0.0123$) or with cells cultured with FGF-10 ($p < 0.0001$); the difference between control cells and those cultured with FGF-1 approached significance ($p = 0.0553$). Cells from a single preparation of rMSCs cultured through P10 were harvested, loaded into cubes, and implanted. The results were consistent with those for P7 data, with average cube scores of 0.5, 1.4, and 2.2 for control, FGF-1, and PDGF, respectively.

In vitro osteogenesis

Rat MSCs. It has been previously reported that MSCs treated with OS (dexamethasone, ascorbic acid, and B-glycerophosphate) exhibit higher calcium content and more intense von Kossa staining than sister cells cultured without dexamethasone in assays conducted on cells cultured 21 or

more days in a given passage.²² Thus, in these experiments, calcium values measured for OS cultures without growth factors (C) and those treated with both concentrations of FGF or with PDGF were higher than for the same groups in control medium (i.e., no dexamethasone) (Fig. 7; Table 5). For both P2 and P7, the difference in calcium content between OS and non-OS cultures was significant for each treatment group ($p < 0.002$; Fig. 7). For P2 rMSCs, for example, there was a 22-fold (PDGF) to 37-fold (control) increase in average calcium content with the addition of OS. Higher calcium values were also detected in the OS groups than in the no-dexamethasone (no OS) groups in P7 cultures, but the fold increase was not as great as for P2. This may be because calcium content in P7 non-OS cultures was higher than for comparable cultures at P2; the fact that the average calcium content for OS cultures in each treatment group was, conversely, lower than for the same groups in P2 (except for the PDGF-treated cells) was also a factor in the lower fold increase. Notably, the calcium content for rMSCs treated with FGF-10 was significantly lower than for any of the other OS groups at P7.

Among cultures treated with OS, second passage cells treated with PDGF had significantly lower calcium content than for cells cultured without growth factors ($p < 0.003$)

FIG. 6. Effect of FGF-2 and human PDGF on *in vivo* bone formation for rat MSC. Bars indicate the average histologic scores for cell-loaded cubes harvested 6 weeks after implantation. Scores are based on the amount of bone found in cube pores. Rat MSCs had been cultured in control medium (vertical lines), in medium supplemented with FGF-2 at 1 (black bars) or 10 ng/mL (open bars), or with PDGF (10 ng/mL) (diagonal lines). Error bars indicate standard deviation. Scores for cubes loaded with rMSCs treated with FGF-2 or PDGF were significantly higher than those for the other two treatment groups at P4 (CI=95%) and at P7 (CI=99%).

TABLE 3. RAT MESENCHYMAL STEM CELLS IN CERAMIC CUBES *IN VIVO*: SUMMARY STATISTICS FOR BONE FORMATION SCORES BY TREATMENT AND PASSAGE

Passage	Condition	N	Mean	SD	Cell preps	Model-based mean	SE
1	Control	28	2.02	1.22	7	2.03	0.15
	FGF (1)	17	1.80	0.85	7	1.75	0.19
	FGF (10)	24	1.00	0.82	6	1.03	0.16
	PDGF	29	1.84	0.87	7	1.84	0.15
4	Control	31	0.64	0.78	7	0.63	0.14
	FGF (1)	15	1.33	0.68	3	1.24	0.20
	FGF (10)	24	0.13	0.16	6	0.16	0.16
	PDGF	32	1.21	0.75	7	1.20	0.14
7	Control	33	0.19	0.62	7	0.19	0.14
	FGF (1)	20	1.30	0.60	4	1.26	0.18
	FGF (10)	26	0.14	0.36	6	0.17	0.16
	PDGF	35	1.14	0.78	7	1.14	0.14

or for those treated with either concentration of FGF ($p < 0.0001$); those treated without growth factors had calcium levels that were significantly lower than for FGF at 1 ($p = 0.0006$) or 10 ng/mL ($p = 0.03$). Both of these results differ from the results of the *in vivo* ceramic cube assay for second passage rMSCs, where bone formation for cubes loaded with cells treated with FGF-1 and PDGF was comparable to control cultures, and bone development in cubes loaded with rMSCs treated with FGF-10 was significantly lower than for the other treatment groups ($p < 0.0001$). For P7 cells in OS medium, calcium content was lower in cultures treated with FGF-10 than for the other treatment groups ($p < 0.0001$), while calcium content for control cultures was lower than for cells cultured with FGF-1 ($p = 0.0074$) or PDGF ($p = 0.0017$). In this study, the effect of FGF-10 is comparable to the reduced *in vivo* bone formation seen at P7 with comparable cells. However, P7 calcium content for control conditions was comparable to those treated with FGF-1, or with PDGF, while *in vivo* bone formation for

control P7 rMSCs was significantly lower than for those two groups. Results for the calcium assay are, however, consistent with von Kossa staining of rMSCs. Representative P2 and P7 rMSC cultures stained by the von Kossa method are shown in Figure 8. Little, if any, positive staining is seen in the no-dexamethasone cultures at either passage. P2 cultures, with or without FGF or PDGF, received OS stained positively, as indicated by the presence of brown to black bone nodules. The most intense staining was seen for cultures supplemented with FGF-1, although use of FGF-10 also resulted in positively stained nodules. P7 rMSCs in OS medium also stained positively, except for those that were cultured in FGF-10. Negative staining for rMSCs at the higher FGF concentration is consistent with the low calcium values and diminished *in vivo* bone formation for these cells. Staining of other P7 OS cultures, while positive, was less intense than for comparable conditions in P2.

Taken together, the calcium data and von Kossa staining observations are, for the most part, consistent with the

TABLE 4. RAT MESENCHYMAL STEM CELLS IN CERAMIC CUBES *IN VIVO*: STATISTICALLY SIGNIFICANT DIFFERENCES DETERMINED FOR CERAMIC CUBE SCORES IN DIFFERENT MEDIA CONDITIONS FOR CELLS IN PASSAGES 1, 4, AND 7

Passage	Comparison of conditions	Adjusted p-value	Treatment	Comparison of passages	Adjusted p-value
1	Control vs. FGF (1)	1.0000	Control	1 vs. 4	0.0001
	Control vs. FGF (10)	<0.0001		1 vs. 7	0.0001
	Control vs. PDGF	1.0000	FGF (1)	4 vs. 7	0.0470
	FGF (1) vs. FGF (10)	0.0127		1 vs. 4	0.1458
	FGF (1) vs. PDGF	1.0000		1 vs. 7	0.1236
	FGF (10) vs. PDGF	0.0004		4 vs. 7	1.0000
4	Control vs. FGF (1)	0.0553	FGF (10)	1 vs. 4	0.0002
	Control vs. FGF (10)	0.1104		1 vs. 7	0.0001
	Control vs. PDGF	0.0123	PDGF	4 vs. 7	1.0000
	FGF (1) vs. FGF (10)	0.0001		1 vs. 4	0.0021
	FGF (1) vs. PDGF	1.0000		4 vs. 7	1.0000
	FGF (10) vs. PDGF	<0.0001		4 vs. 7	1.0000
7	Control vs. FGF (1)	<0.0001			
	Control vs. FGF (10)	1.0000			
	Control vs. PDGF	<0.0001			
	FGF (1) vs. FGF (10)	<0.0001			
	FGF (1) vs. PDGF	1.0000			
	FGF (10) vs. PDGF	<0.0001			

Adjusted p -values were calculated using a Bonferroni correction for three pairwise comparisons. Adjusted p -values less than 0.05 are statistically significant at overall significance level (Family wise error rate) of 0.05.

FIG. 7. Calcium content for rMSCs at the end of passage 2 and 7. Cultures in either control medium (C) or medium containing OS were augmented with FGF-2 at 1 (black bars) or 10 ng/mL (open bars), PDGF (10 ng/mL) (diagonal lines), or with no additional supplements (control, vertical lines). Average calcium content $\pm$ standard deviation is indicated on the vertical axis. For rMSCs in OS medium in P2, calcium content for rMSCs in medium containing 1 ng/mL FGF was significantly higher than for cells in control medium, while calcium content for cells augmented with PDGF was significantly lower than for cells in the other treatment groups. OS, osteogenic supplement.

results of the *in vivo* osteogenic assay, except for the differences just noted. In addition, bone formation in ceramic cubes for rMSCs without FGF or PDGF decreases dramatically with increased passage number. For example, ceramic cube scores for control cells at P4 and P7 are 70% and 90% lower, respectively, than scores for P1 control cells (Table 2). In contrast, *in vitro* calcium content for P7 control rMSCs is only 30% lower than for P2 cells.

Discussion

Previous reports by our laboratory have described the mitogenic and chondro-stimulatory effects of FGF-2 on hMSCs¹⁰ and the enhanced osteoinductive effect of FGF-2 on rMSCs.⁸ The work described in this study demonstrates that FGF-2 also enhances the ability of hMSCs to differentiate into bone and confirms the mitogenic activity of FGF-2 and

FIG. 8. *In vitro* osteogenic differentiation of rMSCs. von Kossa-stained cultures of rMSCs at the end of second and seventh passage. Mineralization is indicated by brown or black stain. As indicated on the left margin, cells had been cultured in one of two medium conditions: control (C) (containing only ascorbate acid-2 phosphate) or OS (ascorbic acid, dexamethasone, and β -glycerophosphate). For each of the two groups, medium was further augmented with FGF-2 at 1 or 10 ng/mL, PDGF at 10 ng/mL, or with no growth factors (control). Color images available online at www.liebertpub.com/tea

TABLE 5. CALCIUM CONTENT ($\mu\text{g}/35\text{-MM DISH}$) DETERMINED FOR rMSC CULTURES GROWN IN OSTEOGENIC CONDITIONS (OSTEOGENIC SUPPLEMENTS)

Passage	2				7			
Condition	Control	FGF (1)	FGF (10)	PDGF	Control	FGF (1)	FGF (10)	PDGF
Mean	226.82	291.67	266.57	172.60	161.89	199.60	48.60	207.25
SD	18.86	9.52	20.14	13.16	14.07	19.02	4.21	8.32

PDGF on these cells. Thus, hMSCs culture expanded with FGF-2 proliferate more rapidly than cells in control medium and are superior to the latter in their ability to differentiate into bone and cartilage. While hMSCs also proliferate more rapidly in PDGF-containing medium than in control medium, bone formation *in vivo* is not significantly enhanced. It has been reported that FGF-2 in lower doses can increase bone formation *in vivo*,²³ and a role of FGF-2 in MSC osteoblast proliferation and differentiation has been demonstrated.^{24,25} This is the first time that is shown a priming effect of FGF-2 on MSCs to augment their *in vivo* osteogenic potential, similar to previous works showing enhanced chondrogenesis when MSCs are cultured in the presence of FGF-2.⁹

The time and duration of exposure of rat and human MSCs to FGF-2 and PDGF in this study are the same as those described before^{9,10}; that is, cell cultures were exposed to the growth factors at the time of the first change of medium in primary culture and were maintained in these conditions throughout their time in monolayer (undifferentiated) culture. FGF-2 was not present in chondrogenic pellet medium in the Solchaga *et al.* study, but was present in culture medium during *in vitro* osteogenic differentiation in this study. FGF-2 was not, however, present in cell suspensions used to load ceramic cubes.

Previous works have shown that FGF-2 can both increase the proliferation and osteogenic potential of rMSCs when applied exogenously during expansion. However, continuous use of FGF-2 during *in vitro* differentiation completely blocks rMSC osteogenesis.²⁶ This clearly demonstrates that the concentration and timing of FGF-2 stimulation has to be strictly controlled during *in vitro* culture. It has been suggested that addition of FGF-2 appears to maintain the osteoprogenitor cells in a proliferative state delaying or inhibiting subsequent differentiation if continuously administered during differentiation.²⁷

While the most of the results of this study with respect to rMSCs are similar to those reported by Hanada *et al.*,⁸ the details of the procedures were different. In their study, rMSCs were exposed to FGF-2 (with or without BMP-2) at a concentration of 2.5 ng/mL for only 6 days of culture in P1 and not in primary culture. *In vitro* and *in vivo* osteogenesis assays were conducted thereafter without the presence of FGF-2. Increases in cell proliferation and in bone formation in both assays were reported. While the scores for bone formation in ceramic cubes were lower than for this study, the difference may be attributed to the quality of serum rather than time of exposure to FGF-2. The main difference between the two studies, however, is that in this study we maintained rMSCs through seven passages and documented increases in cell proliferation and *in vivo* (except for P1) and *in vitro* osteogenesis throughout the time in culture, while the previous study was limited to rMSCs in P1.

The reasons for the differences for *in vivo* osteogenesis at the end of P1 are not clear (Hanada *et al.* reported a significant increase in bone formation for cells cultured in FGF-2, while we did not). Also unclear, as indicated above, is an explanation of why this study demonstrated significant differences for *in vitro* calcium deposition but not for *in vivo* bone formation among the different treatment groups for first passage rMSCs.

While there are many similarities in the effects of FGF-2 and PDGF on human and rat MSCs, there are also notable differences. For example, both growth factors stimulated the proliferation of both rat and hMSCs. For hMSCs, however, the effect of FGF-2 is more pronounced compared with PDGF, and, in fact, use of FGF-2 resulted in a greater increase in the number of cumulative population doublings than did PDGF in each of the seven experiments that were conducted. For rMSCs, use of PDGF resulted in greater cell proliferation than for FGF-1 in three of the five experiments in which FGF was tested at this concentration. The mitogenic effect of either growth factor, however, was much greater for hMSCs than for rMSCs. For example, for hMSCs, the average increase in cumulative population doublings at P7 was 8.8 doublings for FGF and 4.5 for PDGF. Respective increases for these factors for rMSCs are only 1.57 and 1.71. This increase in hMSC proliferation has important implications for possible tissue engineering applications. The data from the hMSC preparation whose population dynamics are illustrated in Figure 3, for example, indicate that the cumulative cell yield per primary culture dish at the end of P7 was 5.38 billion, 61.2 billion, and 890 billion cells for control, PDGF-treated, and FGF-treated hMSCs, respectively. Thus, there is a greater than 15-fold increase in the number of available cells at this passage when they are cultured with FGF instead of FBS alone. More importantly, those cells also have greater potential to differentiate into bone than do cells cultured in FBS alone.

Although FGF-10 was found to be the optimal concentration for proliferation and for osteogenic and chondrogenic differentiation of hMSCs, this concentration was too high for rMSCs, and a concentration of 1 ng/mL was selected instead. Proliferation of rMSCs in FGF-10 did not differ from that seen with FGF-1 in most of the cell preparations nor did morphological changes or differences in calcium content and von Kossa staining appear in first passage rMSCs. However, *in vivo* bone formation lower than for control cells was detected as early as P1 for rMSCs maintained in the higher FGF-2 concentration, and bone formation as a percentage of control declined from 49% for P1 to 20% for P4 rMSCs. Concomitant with these declines for *in vivo* bone formation, *in vitro* calcium content and von Kossa staining also decreased for P5 and P7 rMSCs cultured in FGF-2 at the higher concentration.

The results of this study show that hMSCs proliferate more rapidly than rMSCs for any of the media conditions (control,

FGF-2, PDGF) tested. However, scores based on the amount of bone in ceramic cubes implanted in host animals are higher for rMSCs than for hMSCs. The osteoinductive potential of PDGF has been demonstrated *in vivo* and *in vitro*.^{28,29} The comprehensive effect of PDGF on MSC osteogenic differentiation hasn't been explored before.

Although there are numerous details that require further discussion, the overall findings are that lower passage numbers preserve the multipotential differentiation capacity of MSCs. In addition, an earlier report by our group showed that hMSCs kept in P1 for as long as 60 days produced more bone in ceramic cubes than sister cells subcultured every 7 days.³⁰ The addition of FGF-2 to growth medium preserves the compact morphology of the hMSCs and enhances their ability to differentiate along different pathways while also enhancing their proliferative capabilities. This alone has importance for tissue engineering logic since the scale of such tissues in humans requires very large quantity of cells and, to be successful for fabricating tissues *ex vivo*, enhanced receptivity to phenotypic induction is very important. These studies have not approached the mechanisms behind enhanced proliferation coupled with enhanced differentiation, but it is clear that these are separate activities. The comparisons between rMSCs and hMSCs and the lack of optimization of condition for rMSCs clearly emphasize the separation of proliferation and differentiation. Future studies will focus on the mechanism governing these pathways.

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