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EFFECT OF SERUM ON HUMAN BONE MARROW STROMAL CELLS: EX VIVO EXPANSION AND IN VIVO BONE FORMATION

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Background. Bone marrow stromal cell (BMSC) transplantation may offer an efficacious method for the repair of bone defects. This approach has been developed using BMSCs expanded *ex vivo* in medium with fetal bovine serum (FBS). For clinical applications, however, contact of BMSCs with FBS should be minimized. We studied the effect of FBS substitutes on both human BMSC proliferation *in vitro* and subsequent bone formation *in vivo*.

Methods. BMSC proliferation was measured by colony forming efficiency (CFE) and by cell numbers at consecutive passages. Bone formation was studied in 6- to 8-week-old transplants of human BMSCs in immunocompromised mice.

Results. Medium with FBS was more effective in stimulating BMSC proliferation than medium with either human serum (HS) or rabbit serum (RS). Compared

to bone formed by BMSCs cultured continuously with FBS, bone formed by cells cultured with HS, or with FBS switched to HS, was considerably less extensive, while bone formed by cells cultured with FBS switched to serum-free medium (SFM) was considerably more extensive. The increase in bone formation was due to neither the SFM components nor to the proliferation status of BMSCs prior to transplantation.

Conclusions. Our data demonstrate that for *ex vivo* expansion of human BMSCs, medium with FBS remains most effective. However, incubation of human BMSCs in SFM prior to *in vivo* transplantation significantly stimulates subsequent bone formation. This finding increases the practicality of using culture-expanded BMSCs for autologous human transplantation and suggests the presence of osteogenic inhibitors in serum.

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When culture-expanded bone marrow stromal cells (BMSCs) are transplanted *in vivo* into an open system (that is, one in which host tissues have free access to the transplant), rapid vascularization of the transplant is followed by

osteogenesis. Transplanted heterotopically into either syngeneic or immunocompromized recipients, BMSCs of mouse (1–3), rat (4–6), rabbit (7, 8), dog (9), and human (3, 10–14) origin form an extensive bone. Transplanted orthotopically, syngeneic BMSCs can regenerate critical-size bone defects, such as in the femora of rats (15) and in the calvariae of mice (16). Moreover, in immunocompromized animals, the two latter types of bone defects can be healed by human BMSCs (17); [Mankani et al., in preparation]. In some preliminary clinical studies, autologous human BMSCs have been transplanted into long bone and mandibular defects (18, 19). These combined data support the concept that BMSC transplantation can be utilized in the repair of large bone defects caused by extensive trauma, tumor removal, bone infection, congenital malformation, or fracture non-union.

BMSC transplantation depends upon their *ex vivo* expansion from a limited volume of aspirated bone marrow for the generation of large numbers of cells. For this purpose, all previous transplantation studies have used cultures in a medium containing fetal bovine serum (FBS). For widespread clinical application, however, contact of BMSCs with FBS must be minimized because FBS has been implicated as a cause of delayed hypersensitivity reaction (20) and as a potential vector for prion transmission (21). To solve this problem, we have investigated the efficacy of FBS substitutes for both human BMSC proliferation *in vitro* and bone formation *in vivo*. We demonstrate that FBS-containing medium remains most efficient for *ex vivo* expansion of human BMSCs. In contrast, substitution of FBS-containing medium for serum-free medium several days before BMSC transplantation is not only permissive but in fact, quite stimulatory for subsequent bone formation *in vivo*.

MATERIALS AND METHODS

Bone marrow donors. Fragments of bone with marrow were obtained from six patients undergoing reconstructive surgery under IRB approved procedures, in accordance with NIH regulations governing the use of human subjects under protocol 94-D-0188. Donor information is summarized in Table 1.

Bone marrow cultures. Fragments of trabecular bone and marrow were scraped gently with a steel blade into cold α -modified Minimum Essential Medium (α MEM, Life Technologies, Grand Island, NY) and pipetted repeatedly. The released marrow cells were passed consecutively through 16- and 20-gauge needles and filtered through a 70- μ m pore size nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove cell aggregates.

Bone marrow single cell suspensions were plated at either low or high cell density: 1.0×10^5 nucleated cells per 25-cm² flask, and 1.0×10^7 nucleated cells per 75-cm² flask (both Becton Dickinson, Lincoln Park, NJ), respectively. Two types of growth medium were used. Serum-free medium (SFM) included α MEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate (Biofluids, Rockville, MD), 10^{-8} M dexamethasone (Sigma), and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (Wako, Osaka, Japan), with or without 0.5% ITS⁺ (Collaborative Biomedical Products, Bedford, MA; per 20 ml: insulin, 12.5 mg; transferrin, 12.5 mg; selenious acid, 12.5 μ g; BSA, 2.5 g; linoleic acid, 10.7 mg). Serum-containing medium consisted of SFM (without ITS⁺) with 20% FBS (Life Technologies, Grand Island, NY), or 20% HS blood type AB (Pel-Freez, Brown Deer, WI), or 20% RS (Life Technologies). Two lots each of FBS and HS and a single lot of RS were used. No significant differences between the lots of each serum were found; therefore, the lots used in particular experiments will not be specified. Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO₂.

TABLE 1. Bone marrow donors used in the study

Donor	Age (yr), sex	Location	Diagnosis
1	15, F	Spine	Scoliosis
2	13, M	Femur	Blount disease
3	7, F	Iliac crest	Cerebral palsy
4	0.5, M	Iliac crest	Bladder extrophy
5	14, F	Iliac crest	Scoliosis
6	13, F	Spine	Scoliosis

BMSC proliferation assays. BMSC proliferation was studied in primary cultures and in consecutive passages. Primary low-density cultures were prepared in quadruplicate and harvested on days 9 to 11. The cultures were fixed with absolute methanol and stained with an aqueous solution of saturated methyl violet (Sigma). Colonies containing 50 or more BMSCs were counted using a dissecting microscope, and colony forming efficiency (CFE, number of BMSC colonies per 1×10^5 nucleated marrow cells) was calculated.

For generation of large numbers of BMSCs, high-density cultures were prepared in triplicate; the medium was replaced on day 6 or 7. The cultures were first passaged on day 12 with two consecutive applications of 1x trypsin-EDTA (both Life Technologies) for 5–10 min each at room temperature. The cells harvested from each 75-cm² flask were counted separately using a Coulter Counter (Coulter Electronics, Inc, Hialeah, FL) and plated into a separate 175-cm² flask. The second passage was performed 4 days later, and cells were again counted.

To estimate the fraction of proliferating cells at the time of transplantation, cultures in 8-well chamber slides (Nalge Nunc International, Naperville, IL) were prepared. The same cell densities and culture conditions were used as in corresponding cultures designated for transplantation. Just before the latter were harvested for transplantation, the chamber slide cultures were labeled with bromodeoxyuridine (BrdU) labeling reagent (Zymed Laboratories, Inc., South San Francisco, CA), according to the manufacturer's recommendations. To ensure that the majority of proliferating BMSCs have incorporated the label, a 20-hr exposure was chosen which is equal to the average duration of the BMSC mitotic cycle *in vitro* (22). BrdU incorporated into proliferating cells was detected using a biotinylated monoclonal mouse anti-BrdU antibody (Zymed BrdU Staining Kit), and visualized using a streptavidin-biotin staining system, according to the manufacturer's recommendations. For each cell density and culture condition, 10 microscopic fields (objective 20x) were randomly chosen, and 100 cells per field, labeled and unlabeled with BrdU, were counted.

Analysis of variance was performed and post test comparison was done using the Bonferroni multiple comparison test (InStat, GraphPad, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

Assay of bone formation *in vivo*. BMSCs of the third to fifth passages were used for the *in vivo* transplantation assay. The purpose and design of each transplantation experiment are summarized in Table 2. In some groups, the original medium was replaced with a medium of a different composition 3 days before transplantation.

The transplantation technique was performed as described in detail elsewhere (3). Briefly, trypsinized and pelleted BMSCs were resuspended in 1 ml of the same medium in which they had been cultured prior to harvest. As an exception, in experiment 4, group 2, BMSCs that had been cultured in medium with FBS were washed three times with SFM and resuspended in SFM (without ITS⁺). The cell suspensions were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (particle size 0.5–1.0 mm, generously provided by Zimmer, Inc., Warsaw, IN), and the mixtures were incubated at 37°C for 90 min with slow rotation (25 rpm). The particles with attached cells were collected by a brief centrifugation and incorporated into a secondary matrix. Each transplant was consecutively mixed with 15 μ l of mouse fibrinogen (3.2 mg/ml in

TABLE 2. Experiments studying bone formation in vivo

Exp	Donor	Purpose of the experiment	Culture conditions			No. of transplants	BMSCs per transplant, $\times 10^6$					
			Group	Original medium	Secondary medium ^a							
1	1	To study the effect of different sera or serum supplements on subsequent bone formation	1	FBS	FBS	3	2.5					
			2	HS	HS	3						
			3	FBS	HS	3						
			4	FBS	SFM with ITS ⁺	3						
2	2	To verify the results of exp. 1; to investigate the effect of RS; to study the potential influence of ITS ⁺ components on subsequent bone formation	1	FBS	FBS	5	1.5					
			2	HS	HS	5						
			3	FBS	HS	5						
			4	FBS	SFM with ITS ⁺	5						
			5	RS	RS	4						
			6	FBS	FBS + ITS ⁺	5						
			7	FBS	SFM (w/o ITS ⁺)	5						
3	5	To study the influence of BMSC proliferation status just before transplantation on subsequent bone formation	All groups	FBS	FBS		2.4					
			1	Cultures confluent prior to transplantation	5							
			2	Cultures 70% confluent prior to transplantation	4							
			3	Cultures 30% confluent prior to transplantation	5							
			6	1	Cultures confluent prior to transplantation	5		1.9				
				2	Cultures 70% confluent prior to transplantation	4						
				3	Cultures 30% confluent prior to transplantation	5						
			4	6	To study whether an extensive washing of BMSCs in SFM before transplantation is sufficient to increase subsequent bone formation	All groups		FBS	FBS		2.0	
						5		1	No washing; attachment to vehicles in medium with FBS	4		1.7
								2	Extensive washing of cells with SFM; attachment in SFM	4		
6	1	No washing; attachment to vehicles in medium with FBS				3	2.0					
	2	Extensive washing of cells with SFM; attachment in SFM				3						

^a The original medium was replaced with the secondary medium 3 days before transplantation.

PBS) and 15 μ l of mouse thrombin (25 U/ml in 2% CaCl₂, both Sigma), and a solid fibrin gel was formed. After this procedure, more than 90% of BMSCs were incorporated into the matrices.

Eight- to 15-week-old immunodeficient female beige mice (bg-nu-xid, Charles River Laboratories, Raleigh, NC, or Harlan Sprague Dawley, Indianapolis, IN) were used as transplant recipients. Operations were performed in accordance to specifications of an approved small animal protocol (97-024) under anesthesia achieved by i.p. injection of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) at 140 mg/kg body weight and Xylazine (Butler, Columbus, OH) at 7 mg/kg body weight. A mid-longitudinal skin incision of 1 cm length was made on the dorsal surface of each mouse, and up to four subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket. The incisions were closed with surgical staples.

The transplants were recovered 6 to 8 weeks posttransplantation, fixed and decalcified in Bouin's solution (Sigma), cut into halves, and embedded into a single paraffin block so that the largest surface areas were sectioned. Three sections separated by 100- μ m steps were prepared from each block and stained with hematoxylin and eosin. Bone formation was blindly estimated by three independent investigators using the following semi-quantitative scale: 0, no signs of bone formation; 1+, poor bone formation, a single, small bone trabecula is found in only a few sections; 2+, weak bone formation, bone occupies a small part of each section or of some sections; 3+, moderate bone formation, bone occupies a significant part, but less than one-half of the sections; 4+, abundant bone formation, bone spreads over more than one-half of the sections. A good correlation ($r=0.973$) was found between the results received by bone scoring and by histomorphometry, with a logarithmic relationship existing

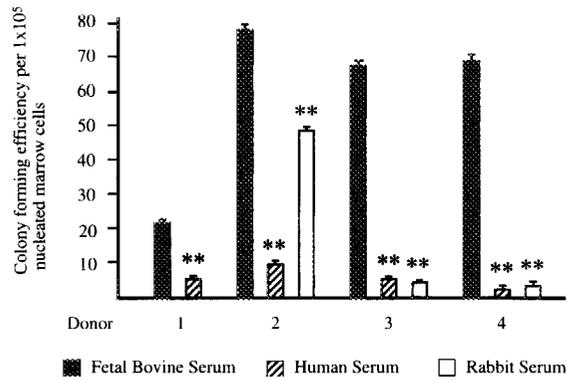


FIGURE 1. Human BMSC colony forming efficiency in primary bone marrow cell cultures in medium with different types of serum. Each column represents an average of four cultures \pm SEM. ** $P < 0.01$ in comparison with FBS-containing cultures.

between the bone scores and the actual bone areas (Mankani MH, unpublished data). The average bone-formation score was calculated for each transplant and for each experimental group. Analysis of variance was performed and post test comparison was done using the Dunnett multiple comparison test (Instat, GraphPad, San Diego, CA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of serum type on BMSC proliferation in vitro. BMSC colony forming efficiency was studied in low-density primary cultures from four donors (Fig. 1). In medium with FBS, the CFE was found to be 21.8 ± 1.2 , 77.3 ± 1.3 , 66.8 ± 1.8 , and 68.0 ± 4.0 per 1×10^5 bone marrow nucleated cells for donors 1 to 4, respectively, which is similar to the normal range observed in our previous studies (23). In medium with HS, the CFE for donors 1 to 4 was 5.8 ± 0.5 , 9.9 ± 0.8 , 5.8 ± 0.5 , and 2.3 ± 0.8 per 1×10^5 bone marrow nucleated cells, respectively. In medium with RS, the CFE for donors 2 to 4 was 48.3 ± 0.8 , 4.5 ± 0.3 , and 3.3 ± 1.7 per 1×10^5 bone marrow nucleated cells, respectively. For each donor, the CFE in medium with either HS or RS was significantly lower than in medium with FBS. No BMSC colonies were formed in cultures in SFM, both with and without ITS⁺.

The numbers of BMSCs harvested at the first and the second passage were counted for donors 1 to 4. In cultures with FBS, the mean cell numbers differed considerably from one donor to another: at the first passage, from $3.1 \pm 0.3 \times 10^6$ to $10.6 \pm 1.1 \times 10^6$ per culture, and at the second passage, from $11.8 \pm 0.4 \times 10^6$ to $28.5 \pm 1.0 \times 10^6$ per culture. For each donor, cultures with FBS yielded significantly more BMSCs than cultures with either HS or RS. At the first passage, the mean numbers of cells harvested from cultures with HS and RS constituted $27.9 \pm 4.2\%$ and $8.7 \pm 1.1\%$, respectively, of the number harvested from cultures with FBS ($P < 0.01$). At the second passage, the corresponding ratios were 15.6 ± 3.6 and $8.4 \pm 2.3\%$ ($P < 0.01$). No BMSC growth was observed in high-density cultures in SFM.

Effect of serum type on bone formation in vivo. Bone formation by transplanted BMSCs was studied in four experiments. The numbers of BMSCs harvested in each experiment at the time of transplantation are summarized in Table 3. Six to 8 weeks posttransplantation, all transplants demonstrated new bone on the surface of HA/TCP particles. Mor-

phological features of the new bone were identical to those described in our earlier publications (3, 13, 24, 25).

In the first experiment, we studied bone formation by BMSCs cultured with and without FBS. Compared to bone formed by cells cultured continuously in medium with FBS (group 1; average bone score 2.8 ± 0.4), bone formed by cells cultured constantly in medium with HS (group 2; 1.5 ± 0.4), or in FBS-containing medium changed to medium with HS (group 3; 2.0 ± 0.2), was considerably less extensive. In contrast, bone formed by cells cultured in FBS-containing medium changed to SFM with ITS⁺ (group 4; 4.0 ± 0.0) was considerably more extensive (Fig. 2).

In the second experiment, groups 1 to 4 represented the same culture conditions as in the first experiment; the extent of bone formed in these groups corroborated our previous results (Fig. 3). In addition, group 5 demonstrated that BMSCs cultured continuously in medium with RS form extensive bone (average bone score 3.6 ± 0.3). In groups 6 and 7, we explored mechanisms by which BMSC incubation in SFM with ITS⁺ stimulated subsequent bone formation. We tested the hypothesis that the ITS⁺ ingredients promote BMSC osteogenic differentiation. In group 6, medium with FBS was replaced with medium containing both FBS and ITS⁺, and in group 7, FBS-containing medium was replaced with SFM without ITS⁺. Contrary to our hypothesis, bone formed in group 6 (average score 2.9 ± 0.5), was not different from controls (group 1, 3.0 ± 0.1), although group 7 demonstrated the highest levels of bone formation (4.0 ± 0.0), significantly more extensive than in the control group (Fig. 3). These results demonstrated that the stimulatory effect of SFM on bone formation was not caused by the ITS⁺ ingredients.

At the time of harvest (at 90–95% confluence), BMSCs cultured continuously in medium with FBS featured abundant mitotic figures, and the fraction of proliferating (BrdU labeled) cells was as high as $75.0 \pm 2.1\%$ and $77.6 \pm 2.0\%$ for donors 1 and 2, respectively. In contrast, cultures incubated for 3 days in SFM without ITS⁺ had virtually no mitotic figures, and the proliferating cell fraction was as low as $3.9 \pm 0.4\%$ (donor 1), and $5.2 \pm 0.7\%$ (donor 2). Based on these observations, we suggested that the extent of in vivo bone formation might depend on BMSC proliferation status just before transplantation. We tested this hypothesis in the third experiment. BMSCs were plated in FBS-containing medium at various cell densities such that at the time of transplantation, cultures of group 1 had reached confluence and showed very few mitotic figures, while cultures of groups 2 and 3 were only 70 and 30% confluent, respectively, and full of dividing cells. The fraction of proliferating cells was, in group 1, 8.6 ± 0.9 and $6.8 \pm 0.4\%$ for donors 5 and 6, respectively; in group 2, it was 79.4 ± 1.9 and $77.8 \pm 1.0\%$, and in group 3, 80.4 ± 2.5 and $76.9 \pm 2.3\%$. Despite the disparate proliferation status, differences between the extent of bone formed in all three groups were, for each donor, not statistically significant (Fig. 4). This experiment demonstrated that it was not the proliferation status of BMSCs before transplantation that determined the extent of subsequent in vivo bone formation.

In the fourth experiment, we tested the assumption that a 3-day incubation of BMSCs in SFM before transplantation might be excessive, and that mere removal of FBS followed by a brief washing with SFM would be sufficient to increase subsequent bone formation. In controls (group 1), the regular

TABLE 3. The actual numbers of BMSCs harvested at the time of transplantation

Exp	Donor	Culture conditions			Number of BMSCs plated, $\times 10^6/\text{flask}^b$	Number of flasks	Time before harvest, days	Number of BMSCs harvested at the time of transplantation, $\times 10^6$ ($\times 10^6/\text{flask}$)	
		Group	Original medium	Secondary medium ^a					
1	1	1	FBS	FBS	5.0	1	4	26.5	
		2	HS	HS	5.0	1		15.6	
		3	FBS	HS	5.0	1		20.0	
		4	FBS	SFM with ITS ⁺	5.0	1		7.5	
2	2	1	FBS	FBS	1.8	1	4	14.0	
		2	HS	HS	3.0	1		9.3	
		3	FBS	HS	2.5	1		13.5	
		4	FBS	SFM with ITS ⁺	5.0	1		9.8	
		5	RS	RS	2.0	1		6.2	
		6	FBS	FBS + ITS ⁺	1.8	1		13.3	
		7	FBS	SFM (w/o ITS ⁺)	7.0	1		12.2	
3	All groups:		FBS	FBS					
		5	1	Confluent cultures		4.0	1	3	16.8
		2	70% confluent cultures		1.0	2		9.4 (4.7)	
		3	30% confluent cultures		0.5	4		11.2 (2.8)	
	6	1	Confluent cultures		4.0	1	3	19.6	
		2	70% confluent cultures		1.0	2		11.1 (5.5)	
		3	30% confluent cultures		0.5	4		14.0 (3.5)	
	4	All groups:		FBS	FBS				
			5	1	No washing; attachment in medium with FBS		3.0	1	3
		2	Washing with SFM; attachment in SFM						
6		1	No washing; attachment in medium with FBS		3.0	1	3	17.5	
		2	Washing with SFM; attachment in SFM						

^a The original medium was replaced with the secondary medium 3 days before transplantation.

^b 175 cm² flasks were used.

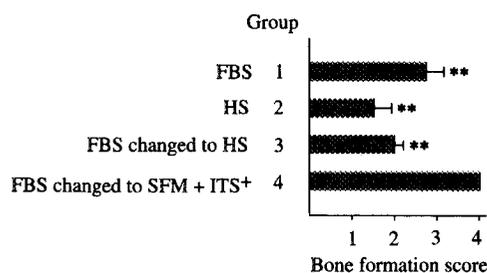


FIGURE 2. Bone formation in vivo by BMSCs from donor 1 cultured under different conditions. Each column represents an average of three transplants \pm SEM. $**P < 0.01$ in comparison with group 4.

procedure was used: BMSCs were both cultured and attached to HA/TCP particles in medium with FBS. In group 2, BMSCs harvested from the same cultures were washed three times with SFM and attached to the vehicles in SFM. Results of this experiment are shown in Figure 5. For donor 5, bone formed in group 2 (average score 1.3 ± 0.4) was significantly less extensive than in control group (3.9 ± 0.1); for donor 6, bone formed in group 2 (3.3 ± 0.2) was not different from controls (3.7 ± 0.2). Thus, short incubation of trypsinized BMSCs in SFM before transplantation was not sufficient to increase subsequent in vivo bone formation.

DISCUSSION

In this study, we compared the effect of different sera and serum-free media on human BMSC proliferation in vitro and subsequent bone formation in vivo. Our most essential findings demonstrate that human BMSCs incubated in serum-free media for several days before transplantation form significantly more bone than BMSCs cultured continuously in serum-containing media (with either FBS, HS, or FBS switched to HS). The increase in osteogenic potential of BMSCs cultured without serum is not caused by the components of ITS⁺ in SFM, or by the state of BMSC proliferation before transplantation. Our data instead suggest the presence of factors in serum (at least, in FBS and HS) that inhibit osteogenic differentiation of human BMSCs. Although RS has not been shown to have such an influence on human BMSCs, its effect on rabbit and mouse BMSCs is worth studying.

Each BMSC colony formed in low cell density cultures originates from a single precursor cell referred to as the colony forming unit-fibroblast (CFU-F) (26, 27). In steady-state conditions in vivo, most CFU-Fs are in G₀ stage of the cell cycle (26), and enter into S phase between 24 and 60 hr after plating (7). The BMSC colony-forming assay therefore reflects the number of CFU-Fs entering into proliferation, as well as the ability of immediate CFU-F descendants to sus-

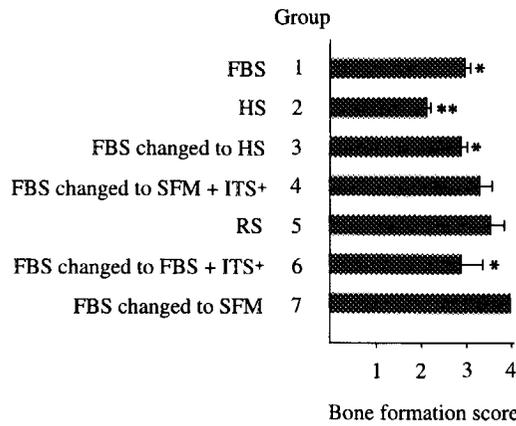


FIGURE 3. Bone formation in vivo by BMSCs from donor 2 cultured under different conditions. Each column represents an average of 4 or 5 transplants ± SEM. *P < 0.05, **P < 0.01 in comparison with group 7.

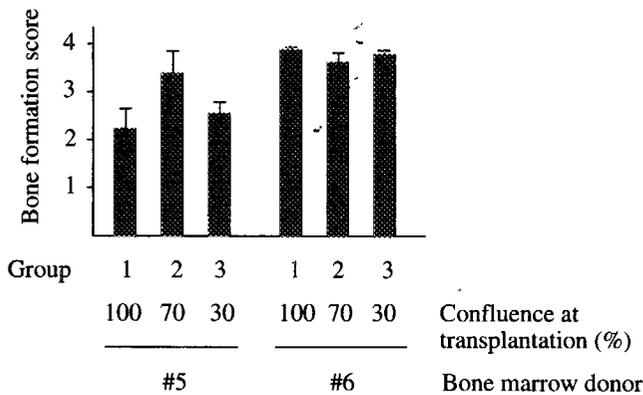


FIGURE 4. Bone formation in vivo by BMSCs from donors 5 and 6 harvested at various degree of confluence. Each column represents an average of four or five transplants ± SEM. Differences between groups from each donor are not statistically significant.

tain proliferation. A separate assay counts BMSCs harvested at the consecutive passages; it reflects the proliferation rate of more remote CFU-F descendants. In both of these assays, human BMSCs demonstrated much more rapid proliferation in medium with FBS than in medium with either HS or RS, while no BMSC proliferation occurred in SFM. Our data therefore indicate that to date, FBS remains the most efficacious additive to induce and sustain BMSC proliferation in vitro. Although other experimental procedures can establish BMSCs in serum-free or semi-serum-free conditions (28, 29), they are not currently amenable for the large scale ex vivo BMSC expansion required for therapeutic tissue regeneration.

In contrast to our data, another group reported that passaged human BMSCs proliferated faster in medium with HS than in medium with FBS by a factor of 1.25 to 1.7 (30). In their cultures with FBS, human BMSC CFE was 2.4 ± 1.4 (31) or 2.75 ± 1.45 (32) per 1×10^5 nucleated bone marrow cells, which is 10 to 30 times lower than in our cultures. The difference could be caused by the use of suboptimal FBS lots in those studies; this would also explain why, in their hands,

medium with FBS supported less BMSC proliferation than medium with HS.

In our earlier study, we attempted to expand bone formation by modifying BMSC transplantation parameters. It has been shown that human BMSC in vivo osteogenic differentiation is very sensitive to the nature of the transplantation vehicle. While some vehicles do not promote any bone formation, the most extensive bone is formed when human BMSCs are transplanted within HA/TCP particles, with or without fibrin as a secondary matrix (3). In our present study, we tried to optimize cultivation parameters by limiting human BMSC contact with xenogeneic serum. We demonstrated that a 3-day incubation of BMSCs in SFM before transplantation significantly stimulates subsequent in vivo bone formation. Future studies should determine periods of serum withdrawal other than 3 days that can increase bone formation even further. Meanwhile, when contact of BMSCs to SFM was limited to washing and short incubation after culture in FBS-containing medium and trypsinization, no increase in bone formation was noted, suggesting that osteogenic stimulation may be a longer process and may require cells to be attached to a substrate.

The results of this study were received using cells derived exclusively from juvenile donors. Previously, it has been demonstrated that both the incidence and the extent of bone formed in transplants of human BMSCs have no correlation with the age of bone marrow donors (3, 10, 11). It seems, therefore, likely that our results could be applied to BMSCs obtained from older, and more clinically relevant, groups of patients.

Heterotopic transplantation has proved to be a sensitive tool for revealing differentiation capacities of BMSCs and related cell types. Upon transplantation, rabbit BMSCs derived from red and yellow marrow formed heterotopic ossicles containing stroma with abundant hematopoiesis, and hypocellular stroma with mainly fat cells, respectively, thus mirroring the composition of the original tissues (8). Human cementum-derived cells formed a unique hard tissue reminiscent of cementum in situ and yet distinct from typical bone (33). BMSCs from transgenic mice deficient for membrane-bound matrix metalloproteinase reproduced impairment of both osteogenic capacity and collagenolytic activity (34). BMSCs from patients with McCune-Albright syndrome recapitulated abnormal features of the fibrous dysplastic lesion of bone, providing an in vivo model of the disease (24). These data show that differentiation potential of BMSCs, both normal and pathological, remains qualitatively unchanged during prolonged expansion ex vivo. It is therefore of interest to note that quantitatively, BMSC osteogenic capacity can be considerably modified by culture conditions, as has been demonstrated in this study.

Depending on the culture conditions, BMSCs can develop certain differentiated features directly in vitro. Among rabbit BMSCs, adipogenic differentiation could be induced with a medium containing rabbit plasma; after the latter had been replaced with FBS, the cells underwent dedifferentiation, losing their adipocytic morphology and acquiring the ability to form bone in vivo (35). In human BMSCs cultured with RS, we, too, observed the development of abundant Oil Red O-positive lipid vesicles (not shown), an evidence of adipogenic differentiation (36). These BMSCs with preadipocytic character remained highly osteogenic: they formed abundant

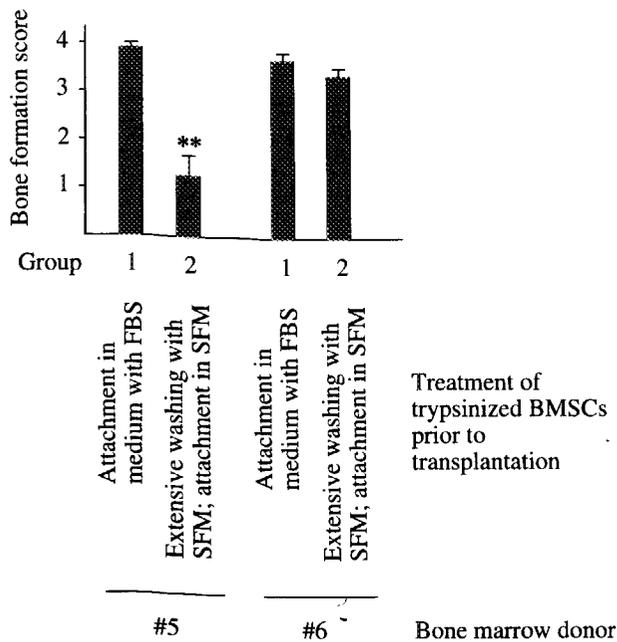


FIGURE 5. Bone formation in vivo by BMSCs from donors 5 and 6 after different pretransplantation regimens. Each column represents an average of three or four transplants \pm SEM. ** $P < 0.01$ in comparison with group 1 from donor 5; differences between groups from donor 6 are not statistically significant.

bone when transplanted directly from the RS-containing cultures. These observations support the concept of the reversibility of BMSC differentiation (35, 37, 38). They also demonstrate that the patterns of BMSC in vitro differentiation, however conspicuous, do not predetermine the direction of their in vivo differentiation; the same conclusion has been reached in studies of mouse BMSC lines (39).

Taken together, our data demonstrate that the most rapid expansion of human BMSCs ex vivo is supported by a medium containing FBS, although the most abundant bone formation in vivo is achieved by BMSCs that have been incubated in serum-free medium for a period preceding transplantation. Our findings provide a way for improving transplant performance although reducing a patient's exposure to the risks incurred by xenogeneic sera; they therefore increase the practicality of using culture-expanded BMSCs for autologous human transplantation. Furthermore, our results suggest the presence of osteogenic inhibitors in serum that may contribute to the appropriate balance of osteogenic and nonosteogenic stromal cell differentiation in the bone marrow microenvironment.

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