Fibroblast Growth Factor-2 Supports *ex Vivo* Expansion and Maintenance of Osteogenic Precursors from Human Bone Marrow*

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ABSTRACT

We have investigated the effects of different growth factors on the proliferation and osteogenic potential of primary cultures of human bone marrow stromal cells (BMSC). Fibroblast growth factor (FGF)-2 was the most effective in promoting growth of these cells *in vitro*. The size of colonies formed in clonal conditions was approximately 2.5 times larger in presence of FGF-2. Also the morphology of BMSC was affected: cells cultured in 10% FCS alone became flattened, whereas FGF-2 expanded cells maintained a fibroblast-like elongated phenotype. Levels of alkaline phosphatase activity in BMSC expanded with FGF-2 were significantly lower (56%) than control and, after stimulation with ascorbic acid, βGlycerophosphate and dexamethasone, FGF-2 expanded BMSC deposited approximately 3-fold more mineralized matrix than control cells. We have assessed osteogenicity of BMSC on hydroxyapatite porous scaffolds (bioceramics) by an ectopic bone formation assay. FGF-2 expanded BMSC yielded a higher bone formation (>20-fold) than control cells. We conclude that FGF-2, promoting BMSC proliferation, maintains cells in a more immature state allowing *in vitro* expansion of human osteo-progenitors which, associated with bioceramics, can differentiate *in vivo* and form bone tissue. (*Endocrinology* **138:** 4456–4462, 1997)

BONE transplantation is an absolute requirement in several pathological conditions: grafts of new tissue are in fact required to achieve a complete recovery for the repair of traumatic lesions, in the reconstructions of segments in plastic surgery or after the removal of neoplasias (1). Material of human (auto or heterologous) or of other species origin has often been used for this purpose (1). Animal tissues have the mechanical properties required. On the other hand, they are a potential source of microbial contaminations, they can elicit immune responses in the recipient, their vascularization and integration in host tissue is not immediate, and finally they have high preparation and storage costs (1). A wide choice of synthetic osteoconductive substitutes is becoming available for specific therapeutic applications (1, 2). Nevertheless, the materials available are not fully satisfactory yet in terms of their osteoinduction, integration within the host tissues, mechanical properties, and lifespan after transplant.

A considerable amount of literature has been published on the therapeutic applications of cells of bone origin (3–5). Several laboratories have shown the potential of bone mar-

row stromal cells (BMSC) in bone reconstitution (6–9). BMSC contain a multipotent self-renewing compartment and are capable of differentiating into several mesenchymal lineages: osteoblasts, chondrocytes, adipocytes, and myocytes (10). When implanted *in vivo*, these cells reconstitute bone and in some conditions cartilage (6, 8, 9, 11). In particular, when combined with mineral containing tridimensional scaffolds, they form a primary bone tissue highly vascularized and colonized by host hemopoietic marrow (8, 11). In this system, the ceramic provides a tridimensional structure, a cell adhesion site, and may act as a primer for the formation of new bone matrix foci; in addition, BMSC differentiate into osteoblasts and deposit extracellular matrix on the ceramic surface.

To be used for clinical therapy, the BMSC system needs to be highly standardized and reproducible in bone formation efficiency. Frequency of bone formation can vary depending on the different human bone marrow primary cultures and strongly depends on cell culture conditions (8). Bone formation was observed only when the cells were cultured in the presence of FBS from highly selected lots, making it necessary to perform laborious serum testing before its use (8).

The effects of growth factors on proliferation and activity of bone cells and marrow stromal cells have been investigated by several groups (12–18).

The possibility to increase BMSC *in vitro* proliferation rate and osteogenic potential has been investigated. We have studied the biological effects of different growth factors and hormones on *ex vivo* BMSC expansion, and here we report culture conditions that represent a significant improvement

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in the technology to expand *ex vivo* human BMSC maintaining their osteogenic potential.

Materials and Methods

Cell culture

Human bone marrow cells were obtained from iliac crest marrow aspirates of healthy donors (one was 7 yr old and 7 ranged in age from 31–42 yr). All the procedures were approved by institutional ethical committee. From a bulk of approximately 1,000 ml of marrow aspirate, ready for bone marrow transplantation, 5- to 10-ml aliquots were taken and centrifuged on a Ficoll separating solution (Seromed, Berlin, Germany) for 20 min at 2500 rpm. The nucleated cells were recovered from the interphase, counted with a nuclear stain (0.1% methyl violet, in 0.1 M citric acid), suspended in Coon's modified Ham's F-12 medium supplemented with 10% FCS (Mascia Brunelli, Milano, Italy), 100 IU/ml penicillin and 100 μg/ml streptomycin (ICN Biomedicals, Costa Mesa, CA), plated in 100-mm dishes at $2–5 \times 10^6$ nucleated cells per dish (corresponding approximately to 1 ml of postFicoll sample and equivalent to $2.6–6.4 \times 10^4$ cells/cm^2) and incubated at 37 C in humidified atmosphere containing 95% air and 5% CO_2. The medium was changed after 4 days and then twice a week. When culture dishes reached confluence (2 weeks after the primary culture), cells were detached with 0.05% trypsin with 0.01% EDTA, counted, and replated in 100-mm dishes at 1×10^5 cells per dish. Each marrow sample was divided into aliquots and placed in culture. Growth factors and hormones were added (when indicated) at the beginning of the cultures. All the growth factors used were human recombinant (Austral Biologicals, San Ramon, CA).

Human skin fibroblasts were obtained from surgical specimens. Samples were minced and plated. Skin fragments were held to the bottom of the culture dishes by sterile coverglasses until a significant cell growth was reached. Fibroblasts were expanded both with and without FGF-2 as described for BMSC.

Colony forming efficiency

To evaluate colony forming efficiency (expressed as ratio between the number of colonies obtained and the number of cells plated), 5×10^5 nucleated bone marrow cells were plated in 60-mm dishes ($\sim 1.8 \times 10^4$ cells/cm^2). The medium was changed after 4 days and then twice a week. After 2 weeks, cells were washed with PBS, pH 7.2, fixed with formaline 4% in PBS, stained with 1% methylene blue in borate buffer (10 mM, pH 8.8) for 30 min and then washed with distilled water. Images of the dishes were acquired by a Cohu black and white camera, digitized by LG-3 frame grabber card (Scion Corporation, Frederick, MD) into a Quadra 840AV Macintosh computer and analyzed with the NIH-Image public domain software, developed at the U.S. National Institutes of Health, and available on the Internet at http://rsb.info.nih.gov/nih-image/. On this platform, a particular computer program (Macro) was integrated to automatically process each dish image: colonies were counted, their total area was calculated, and the average number and size of colonies formed in different culture conditions was derived.

Growth curve

At day 0, 5×10^3 first passage cells were plated in each well of a 24-well plate; cell number was then evaluated at different days via Thiazolyl blue (MTT) staining (19). Briefly, culture medium was removed and replaced with 0.5 ml of medium without FCS; then 25 μl MTT (Sigma, St. Louis, MO) stock solution (5 mg/ml) were added to each culture being assayed. After 3-h incubation, the medium was removed and the converted dye solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 670 nm. Conversion to cell number was based on a standard curve.

Alkaline phosphatase (AP) activity

AP activity was determined as the rate of conversion of p-nitrophenyl phosphate to p-nitrophenol. For each condition to be tested, 2.5×10^4 cells were plated in four 10-mm wells; two wells were washed with PBS

and 100 μl 0.01% SDS were added; after 10 min, a prewarmed solution of 0.5 ml substrate (Sigma catalog no. 104–100) and 0.5 ml alkaline buffer solution (Sigma catalog no. 221) were added in each of the two wells. After a 15-min incubation at 37 C, the contents of each well were added to 10 ml 0.05 N NaOH to quench the reaction and absorbance was read at 410 nm. The remaining two wells were used to perform the MTT assay to normalize AP activity to cell number.

Cells were stained for AP using kit no. 85L-3R (Sigma) and following the direction of the manufacturer. To evaluate the number of AP-positive BMSC colonies, dishes were stained for AP and positive colonies counted. The dishes were then counterstained with hematoxylin, total number of colonies counted, and percentage of AP-positive derived. To evaluate the percentage of AP-positive BMSC expanded in different conditions, at least 300 cells were counted in each of the three independent experiments and percentage of AP-positive BMSC was derived.

In vitro deposition of mineralized matrix

BMSC cultures were expanded both with and without FGF-2. After reaching confluence, cultures were continued for 2 weeks in the absence of FGF-2 and stimulated in medium supplemented with 10% FCS with the addition of 50 μg/ml ascorbic acid (Sigma), 150 μg/ml sodium βGlycerophosphate (Sigma), and 10^{-8} M dexamethasone (Sigma) every other day. After 2 weeks, cells were fixed with formaline 4% in PBS, extensively rinsed in distilled water, stained for 10 min with 2% Alizarin S (pH 4.1), and rinsed twice with absolute ethanol. Quantification of mineralized matrix was performed by computer-assisted analysis on digitized images of the samples. Basal level of mineral deposition was derived from unstimulated cultures and used as threshold to binarize images and to measure the percentage of positive area. Duplicate dishes were processed for MTT assay to normalize data on cell number.

Ectopic bone formation and analysis of the bone volume

The material selected for *in vivo* implantation was a highly porous ceramic support based on 100% hydroxyapatite (HA), of 70–80% porosity. Pore size distribution was: <10 μm, ~3% vol; 10–150 μm, ~11% vol; >150 μm, ~86% vol. The material was produced and kindly provided by Fin-Ceramica Faenza (Faenza, Italy) and dry sterilized for 4 h at 200 C. As control material, in some experiments collagen were used sponges (produced and kindly provided by Coletica, Lyon, France).

In these experiments, only cells from adult donors were used. In our protocol, after 3–4 weeks expansion (corresponding to the first passage), stromal cells were detached from the dishes with 0.05% trypsin and 0.01% EDTA, washed in serum-free medium, and resuspended at 2.5×10^5 cells/20 μl. Two 10-μl aliquots were then applied on two opposite faces of dry ceramic cubes (approximate volume, 64 mm^3), so that each block was loaded with 2.5×10^5 stromal cells. The loading volume was determined on the basis of the average volume of fluid blocks could adsorb. Ceramic blocks were sc implanted just after loading.

When fresh bone marrows were used, nucleated cells were isolated by Ficoll gradient centrifugation, counted, and each cube was loaded with the appropriate number of cells resuspended in two aliquots of 10 μl serum-free medium.

Recipient nude mice (CD-1 nu/nu) of 1 month of age were purchased from Charles River Italia (Calco, Italy), kept in a controlled environment, and given free access to food and water. Mice were cared for and treated according to institutional guidelines. Animals were anesthetized by intramuscular injection of xilazine (1 mg/50 ml) and ketamine (3 mg/50 ml). Grafts were implanted sc on the back of the mice (up to six implants for each animal). Animals were killed 4 and 8 weeks after implantation, grafts removed, and processed for histological analysis. Samples were decalcified (Osteodec, Bio-Optica, Italy) according to manufacturer's instructions, paraffin embedded, sectioned, stained with hematoxylin/eosin, and analyzed for bone tissue using a Zeiss Axiophot microscope (Oberkochen, Germany) and the NIH-Image image analysis software on a Quadra 840AV Macintosh Computer. For each sample, two different depths were analyzed, two histological sections were prepared for each level, and four to six images for each section were acquired to evaluate the amount of bone formation. Bone tissue quantitation was therefore performed for a total area of approximately 70 mm^2 per each sample. The amount of bone formed was assessed as percent of bone *vs.* total tissue, thus excluding the volume occupied by HA.

To determine whether newly formed bone within the ceramic cubes was derived from donor human cells or host mouse cells, tissue sections were stained with the biotinylated human specific BC-2 antitenascin monoclonal antibody (kindly provided by Dr. L. Zardi, Genova, Italy) (20). Mouse bone sections were used as control. Immunoperoxidase staining was performed on paraffin embedded samples as described in Manduca *et al.* (21).

Results

BMSC culture

Human bone marrow cells were prepared as described in the *Materials and Methods* section and plated in a medium containing 10% FCS. In some dishes, different growth factors and hormones were added at different concentrations to study their effect on colony forming efficiency. In particular, the following factors, involved in bone metabolism, were tested: epidermal growth factor (EGF) (1 and 10 ng/ml), platelet-derived growth factor aa (PDGFaa) (1 and 10 ng/ml), platelet-derived growth factor bb (PDGFbb) (1 and 10 ng/ml), basic fibroblast growth factor (FGF-2) (1 and 10 ng/ml), GH (1 and 10 ng/ml), insulin-like growth factor I (IGF-I) (1 and 10 ng/ml), transforming growth factor $\beta1$ (TGF$\beta1$) (2.5 ng/ml), and dexamethasone (Dex) (10^{-8} M). The factor concentration indicated refers to actual amount added and does not consider factors or binding proteins present in the FCS. After approximately 1 week, the first fibroblast colonies derived from colony forming units (CFU-f) could be detected.

To account for the variability in colony number and size observed in cultures derived from marrows obtained from different donors, in each experiment values of the different conditions were normalized to the ones obtained with 10% FCS alone. After 2 weeks in 10% FCS, the average colony number was 20.8 ± 5.5, and the average colony size was 3.2 ± 0.8 mm². Colony number was not affected by most of the factors tested ($P > 0.05$), whereas the addition of FGF-2 resulted in a colony count of 14.6 ± 2.0, corresponding to $30 \pm 0.1\%$ reduction ($P < 0.01$) (Table 1).

Average size of colonies was strongly increased by the addition of EGF (1.74-fold at 1 ng/ml) and especially of FGF-2 (2.48-fold at 1 ng/ml) (Fig. 1). The other factors did not influence significantly BMSC colony size (Table 1).

No major differences in cell density were evident in the colonies in all the conditions tested. Only colonies obtained

TABLE 1. Effect of different growth factors on *in vitro* expanded human BMSC

Condition	Colony no. normalized	Colony size normalized	% AP + colonies
FCS	1	1	47
EGF	1.15 ± 0.1	1.74 ± 0.5	65
PDGFaa	0.81 ± 0.2	0.82 ± 0.1	52
PDGFbb	1.08 ± 0.2	1.16 ± 0.2	50
FGF-2	0.7 ± 0.09	2.48 ± 0.2	31
GH	0.71 ± 0.16	1.36 ± 0.18	52
IGF-I	1 ± 0.28	0.83 ± 0.18	48
TGF-$\beta1$	0.82 ± 0.08	0.65 ± 0.007	22
Dex	1.19 ± 0.19	0.9 ± 0.16	98

Colonies parameters were evaluated as described in *Materials and Methods*. Data represent the mean ± SE of values from three independent experiments. Percentage of AP positive colonies is the average of two independent experiments.
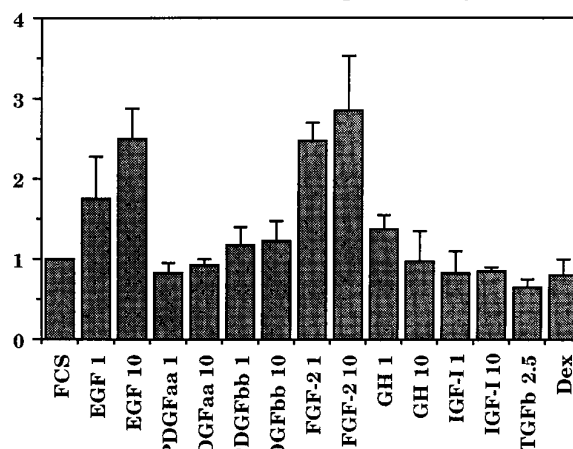

FIG. 1. Human bone marrow CFU-f growth in response to recombinant growth factors and hormones in the presence of 10% FCS. Numbers following factor names indicate the concentration in ng/ml. Dex was used at 10^{-8} M. Data represent the mean ± SE of colony size in duplicate cultures of five independent experiments and are reported normalized to values obtained in the 10% FCS condition (3.2 ± 0.8 mm²). A significant increase ($P \le 0.05$) in clonogenic growth occurred only in response to EGF and FGF-2.

in the presence of Dex displayed a lower cell density and a very spread and flattened phenotype (data not shown).

Colonies obtained with the addition of the different factors were analyzed for AP expression (Table 1). AP-positive colonies increased in cultures treated with EGF and Dex (1.38 and 2.08-fold, respectively) and decreased in cultures treated with FGF-2 and TGF-$\beta1$ (0.65 and 0.46-fold, respectively).

Because FGF-2 was the most active in increasing colony size and was significantly decreasing the percentage of AP positive colonies, we concentrated on its effects on BMSC *in vitro*.

Morphological differences were rather evident in primary cultures between control cells and BMSC cultured with FGF-2 (Fig. 2, a and b). In fact, cells cultured with this factor were thinner and more elongated (b). In the following passages, the original fibroblast-like phenotype was gradually lost by the control cultures which assumed a more flattened cell phenotype (Fig. 2c), whereas it was maintained when FGF-2 was present in the culture medium (Fig. 2d).

Growth curves of BMSC cultured both with and without FGF-2 were derived as described in the *Materials and Methods* section. FGF-2 dramatically increased BMSC growth rate (Fig. 3). At confluence, average cell density was at least 2-fold higher in the cultures treated with FGF-2.

AP activity was colorimetrically quantified on BMSC, expanded either with or without FGF-2, after the first culture passage. The addition of FGF-2 to the culture medium produced significantly ($P < 0.002$) lower AP activity, averaging $56 \pm 0.09\%$ of the control condition. Percentage of AP-positive cells did not vary significantly after expansion between samples in FCS and in FGF-2 (10.88 ± 1.2 and 11.7 ± 1.98, respectively).

In vitro deposition of calcified matrix was investigated. After BMSC expansion to confluence both with and without
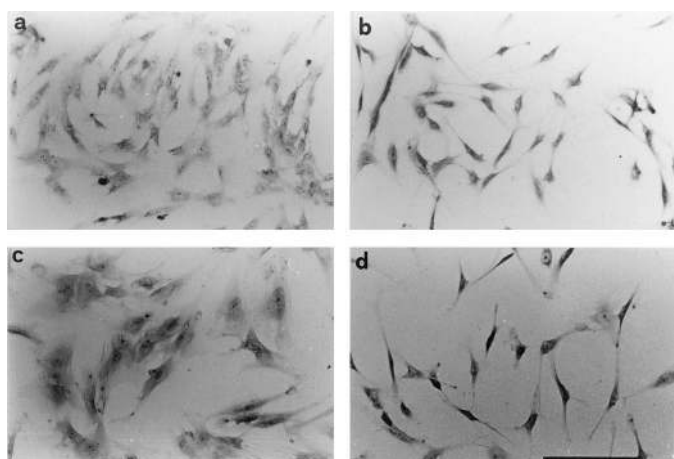
FIG. 2. Comparative morphology of BMSC cultured both with and without 1 ng/ml FGF-2 (primary culture) (a, b) and after 4-week culture (first passage), corresponding to approximately 20 doublings (c, d). BMSC cultured in the presence of FGF-2 (b, d) maintain a stable fibroblastic phenotype, whereas control BMSC in 10% FCS (a, c) progressively assume a larger, more flattened phenotype. *Bar*, 200 μm.
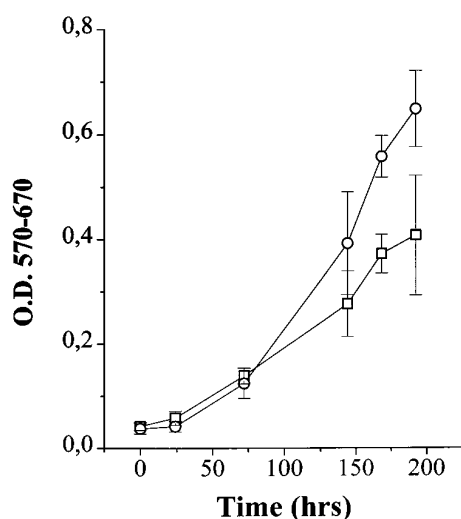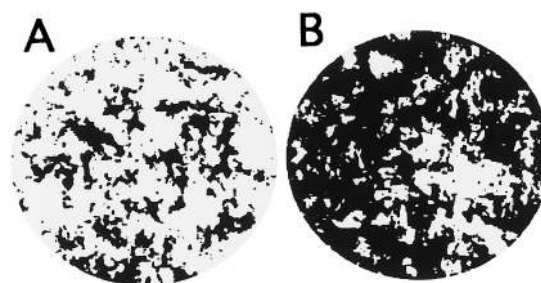

FIG. 4. Representative binarized images of culture dishes showing the mineralized matrix deposited by BMSC expanded without (A) and with (B) 1 ng/ml FGF-2 and then stimulated for 2 weeks with 50 μg/ml ascorbic acid, 150 μg/ml sodium βGlycerophosphate and 10^{-8} dexamethasone. Threshold for binarization has been determined on the basis of background levels displayed in nonstimulated cultures. (B) FGF-2 expanded BMSC yielded approximately three times more mineralized area (70%) than (A) control cultures (23%). Staining: Alizarin S.

TABLE 2. *In vitro* osteogenic potential of human BMSC

| Expansion condition | *In vitro* calcification | | |
	%Ca deposition	Cell no. (norm)	Ca deposition (norm)
10% FCS	14.4 ± 5.2	1	14.4 ± 5.2
10% FCS + FGF − 2	62.4 ± 5.7	1.6 ± 0.1	38.7 ± 2.6

%Ca deposition was derived using binarized images of culture dishes, stained with Alizarin S after a 2-week treatment with ascorbic acid, βGlycerophosphate, and dexamethasone. Cell number was obtained by MTT assay and normalized on values obtained in the 10% FCS condition. Calcium deposition was then normalized on cell number. Data represent the mean ± SE of values from three independent experiments.

found to be significantly higher in FGF-2 expanded BMSC (4.3- and 2.7-fold higher, respectively, before and after normalization for cell number) (Table 2). Comparable results were obtained with von Kossa staining (data not shown).

In vivo osteogenic potential of BMSC

We studied the *in vivo* osteogenic capacity of BMSC expanded with the growth factors tested in the CFE assay. Cells expanded either with or without the above mentioned factors were implanted *in vivo* for an ectopic bone formation assay as described in the methods section. After 4 and 8 weeks, samples were harvested, processed for histology, and evaluated with image analysis techniques. At 4 weeks, bone matrix deposition was detected only in the samples loaded with FGF-2 or Dex expanded BMSC (3.3% and 1.0%, respectively, of the newly formed tissue) (Table 3). At 8 weeks, bone formation reached 12.66% of the total tissue in the case of FGF-2 expanded BMSC and 4.4% in the case of Dex expanded BMSC, it remained below 0.5% in the other conditions tested (Table 3).

Some of the histological sections, stained with hematoxylin/eosin, are shown in Fig. 5. Implants without attached cells were used as controls, and they showed formation of a vascularized loose connective tissue but no evidence of newly deposited bone (Fig. 5a). As an additional control, we implanted HA cubes loaded with human skin fibroblasts cultured with FGF-2; in these samples, we did not detect any bone (data not shown). In our assay, implants with cells

FIG. 3. Growth curves of BMSC expanded in different conditions. At time 0, 5×10^3 first passage BMSCs were plated in each well of a 24-well plate; cell number was then evaluated at different times via Thiazolyl blue (MTT) staining. Reported values are the optical densities obtained by MTT procedure. *Open squares*, 10% FCS; open circles: 10% FCS + 1 ng/ml FGF-2. Data represent the mean ± SE of values of three independent experiments.

FGF-2, cultures were stimulated for 2 weeks with ascorbic acid, βGlycerophosphate and dexamethasone, with no further FGF-2 addition. Dishes were stained with Alizarin S to detect calcium salt deposition, as described in the *Materials and Methods* section. BMSC expanded both with and without FGF-2 were also cultured for the same time without any treatment besides the normal change of medium and used to derive basal levels of Alizarin staining. After 2 weeks of treatment with ascorbic acid, βGlycerophosphate, and dexamethasone, calcium deposition was detected in both BMSC populations, and it was higher when cells were previously expanded with FGF-2. Results were quantified by computer-assisted image analysis (Fig. 4). Mineral deposition was

TABLE 3. Osteogenic potential of BMSC *ex vivo* expanded in the presence of growth factors or hormones

Condition	% Bone 4 weeks	% Bone 8 weeks
FCS	0	<0.5
EGF	0	<0.5
PDGFaa	0	<0.5
PDGFbb	0	<0.5
FGF-2	3.3	12.66 ± 2.05
GH	0	<0.5
IGF-I	0	<0.5
TGF-β1	0	<0.5
Dex	1.0	4.4

The amount of bone formed was derived by quantitative image analysis techniques from histological sections of the samples as percentage of trabecular bone *vs* total tissue formation. All factors were used at 1 ng/ml, TGF-β1 at 2.5 ng/ml and Dex at 10^{-8} M. Data represent the average of values obtained from two independent experiments (average ± SE of six experiments for FGF-2 at 8 weeks).
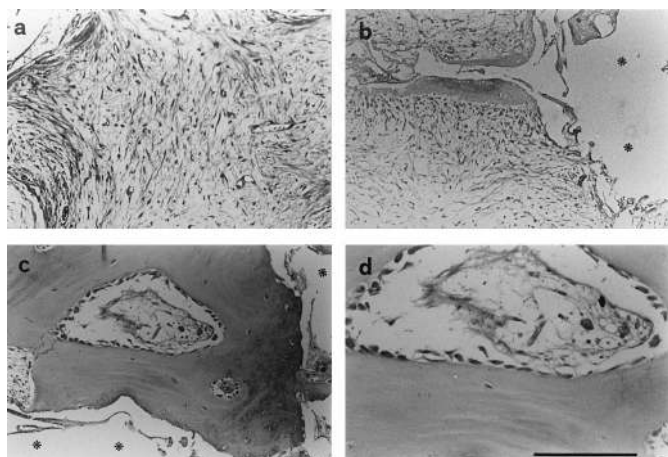

FIG. 5. Representative fields of histological sections of cell specimens ectopically implanted for 8 weeks in nude mice. a, Hydroxyapatite (HA) alone; b, HA loaded with BMSC cultured in 10% FCS; c, HA loaded with FGF-2 expanded BMSC; d, particular of (c) at higher magnification. Empty spaces (*) were occupied by ceramics before decalcification. Bone formation was evident in the samples where BMSC have been implanted onto HA ceramic (b–d); BMSC treated with FGF-2 gave a massive bone formation (c, d). In particular in (c), it is noteworthy to observe deposition of bone matrix from the edges of the ceramic pore toward the center where undifferentiated mesenchymal tissue is present. In (d) osteoblasts lining the edge of bone tissue can be observed. FGF-2 was used at 1 ng/ml concentration. Staining: Hematoxylin-eosin. *Bar*, 200 μm (a–c); 100 μm (d).

expanded in 10% FCS showed little bone matrix deposition (<0.5% of total formed tissue) (Fig. 5b). Newly formed bone was present in nearly all the samples loaded with cells treated with FGF-2 (Fig. 5c); at higher magnification (Fig. 5d), osteoblasts were observed at the edge of the bone tissue, facing mesenchymal, undifferentiated cells. Bone matrix was abundant and osteocytes were enclosed in lacunae. The amount of matrix deposited, quantified using image analysis techniques, varied depending on the primary culture but averaged 12.66 ± 2.05% of the total formed tissue (Table 3). From this percentage, considering the average implant volume (64 mm^3) and the average ceramic porosity (75%), we calculated a total bone formation in 8 weeks of approximately 6.1 mm^3 per implant starting from 2.5 × 10^5 FGF-2 expanded BMSC.

For the *in vivo* experiments, six unrelated primaries expanded with and without FGF-2 were used; bone formation was evident in most of the samples treated with FGF-2 (5/6), whereas only a fraction of untreated samples (1/6) displayed a detectable amount of bone. Interestingly, we never detected any cartilage formation in our samples.

FGF-2 expanded BMSC were also loaded onto collagen sponges and implanted sc in nude mice. Samples were harvested 8 weeks after implantation and processed for histological analysis. These samples displayed only a loose connective tissue but neither bone nor cartilage.

Osteogenic potential of freshly harvested whole bone marrows was also investigated. Samples of freshly harvested bone marrows were adsorbed onto ceramic cubes and sc implanted in nude mice as described in the *Materials and Methods* section. When 5 million nucleated cells (~1 ml fresh bone marrow) were implanted, the amount and characteristics of bone tissue formed were similar to those observed in the experiments where 250,000 expanded BMSC were used. In a similar experiment, 250,000 nucleated cells from fresh bone marrow did not induce any detectable bone formation.

To confirm the origin of the cells within the bone in the ceramic cubes, tissue sections were immunostained with the biotinylated human specific BC-2 monoclonal antibody against tenascin, no counterstaining was performed. The antibody clearly reacted with osteocytes, osteoblasts, and undifferentiated mesenchymal cells of the newly formed bone tissue (Fig. 6B) but did not stain mouse bone cells (Fig. 6C).

Discussion

Bone remodeling and regeneration (*e.g.* during fracture healing) requires the differentiation of new osteoblasts. Therefore, one should consider the existence, also in the adult life, of a stem cell compartment capable of differentiation toward the osteogenic lineage. The bone marrow stromal system (9) is currently believed to be the reservoir of bone stem cells. In particular, the mesenchymal component of bone marrow can support the self repair of bone tissue because it contains a low number of multipotent precursors that are normally quiescent but can become extremely active. These cells can in fact differentiate *in vitro* into several cell lineages (chondrogenic, adipogenic, osteogenic, fibroblastic, myogenic, etc.) (10). Experiments from Friedenstein and colleagues (22), Bab and colleagues (23), and other groups (6, 7, 11, 24) extensively demonstrated the chondrogenic and mostly the osteogenic potential of BMSC *in vivo*. Based on these and similar results, the existence of a mesenchymal stem cell in the bone marrow has been postulated (25). Although this hypothesis appears very attractive, the formal demonstration that a culture derived from a single cell from adult human bone marrow can undergo multiple mesenchymal differentiation pathways has not been proved yet.

In vitro expansion and tissue-specific delivery of autologous BMSC would certainly represent an important advance in skeletal tissue repair. Several reports have shown the therapeutic potential of BMSC in bone reconstitution (3–5). However, to be used for cellular therapy, the BMSC system needs to be highly standardized and reproducible for bone
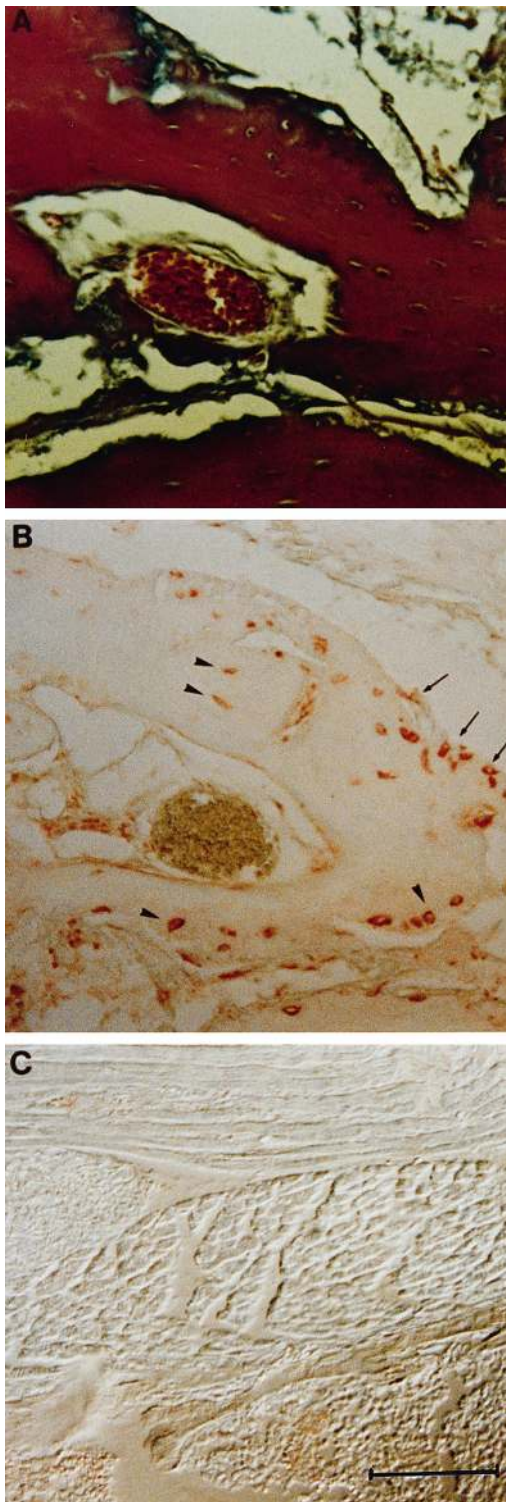
FIG. 6. Immunostaining of paraffin sections of bone tissue generated from grafts of BMSC and ceramics. Paraffin sections of bone tissue generated from grafts of human BMSC/ceramics (A, B) or mouse bone trabeculae (C) were stained with hematoxylin-eosin (A) or with the human specific monoclonal antibody BC-2 against tenascin (B, C). The antibody stains osteoblasts (*arrows*) and osteocytes (*arrowheads*) in the bone formed within the ceramic pores (B). BC-2 antibody does not stain mouse bone cells (C). *Bar*, 50 μm.

formation efficiency. In fact, frequency of bone formation can vary among different human bone marrow primary cultures and strongly depends on cell culture conditions. In the attempt to standardize culture conditions, we have studied the biological effects of different growth factors and hormones on *ex vivo* expansion of BMSC.

Our data suggest that EGF and FGF-2 are the most active in increasing colony size, *i.e.* growth rate, whereas Dex and FGF-2 are the most active in promoting bone formation. Being the most active in promoting both BMSC growth rate and bone formation, FGF-2 has been the only factor selected for further studies. The apparent absence of effect of the other factors (PDGF, GH, IGF, etc.) tested may be explained by the presence in the serum used of the same factors in already maximally stimulatory concentrations. An alternative explanation may be that BMSC secrete these factors in an autocrine fashion.

FGF-2 is a potent modulator of proliferation and activity of bone cells (12) and marrow stromal cells (13–18), although it might not always act directly (13). In our experiments, we noted that the addition of FGF-2 to the BMSC culture medium in the presence of serum yielded a reproducible and constant level of cell proliferation and *in vivo* bone reconstitution. In fact, 83% (5 out of 6) of the primary cultures treated with FGF-2 yielded quantitatively higher amounts of bone as compared with control cultures, where only 1 out of 6 promoted the deposition of detectable bone matrix.

FGF-2 expanded BMSC maintained unaltered their original elongated phenotype for a longer time. This finding may be explained either by a direct effect of FGF-2 on differentiation or commitment level of these cells, or by FGF-2 stimulation of the synthesis and/or organization of extracellular matrix components, which would induce an alteration in the spreading and consequently in the cell phenotype. Interestingly, FGF-2 expanded BMSC expressed lower levels of AP activity (although percentage of AP-positive cells was similar to the one found in BMSC expanded in FCS alone) and displayed *in vitro* a higher osteogenic differentiation potential. These data support the idea that FGF-2 is able to support growth and expansion of osteogenic precursors. Not all factors (*i.e.* EGF) promoting proliferation of the total BMSC population do also maintain the osteogenicity of the expanded cells, thus suggesting that FGF-2 is not only involved in stimulation of BMSC proliferation but also in the maintenance of a particular functional state. It has been described that FGF-2 can substitute for the apical ectodermal ridge to maintain cells in a responsive state required to allow patterning of developing limb (26). It would be interesting to speculate that, in our system, FGF-2 is required to maintain cells in a stem state. Further studies at the clonal level are in progress to understand whether in the presence of FGF-2 certain CFU-f are stimulated or others are inhibited and to determine whether the selected cell population still retains mesenchymal stem potential.

It has been reported that BMSC differentiate *in vitro* in several mesenchymal lineages (10); however, *in vivo* only cartilage and bone formation has been described (6, 8, 11). In our experiments, because we have used an open system (*i.e.* available for vascular ingrowth), we observed only bone tissue. Interestingly, bone matrix deposition was always po-

larized with the newly formed bone facing HA ceramic and the osteoblasts located at the boundary between the newly formed bone and the mesenchymal undifferentiated cells. Bone was never observed when BMSC were implanted on a collagen sponge. We suggest that the mineralized surface of the ceramic may serve as a primer for the initiation of bone matrix deposition.

In agreement with other authors (8, 27), using a monoclonal antibody specific for human tenascin, we have shown that in our implants bone formation was of donor origin.

We also compared the osteogenic potential of BMSC expanded *in vitro* in the presence of FGF-2 *vs.* freshly harvested bone marrow to estimate the osteogenic power of the cells expanded in our conditions. Considering the expansion factor of the cultured BMSC, their osteogenic potential was highly increased with respect to the starting bone marrow sample. This fact *per se* makes it possible to conceive autogenic bone reconstruction in patients without the need for a large bone marrow aspirate, which is always inconvenient and sometimes impossible. We did observe a decrease in the osteogenic efficiency of the expanded BMSC with respect to the fresh bone marrow, but this finding was not unexpected because it is conceivable that during the expansion some of the dividing cells remain in the stem/progenitor compartment, whereas others may start to progress down different lineage pathways. Alternatively, the cotransplanted hemopoietic cells, virtually absent in the cultured stromal cell population, may create an optimal microenvironment (in terms of growth factors and cytokines secreted) for the stromal cells to proliferate and differentiate.

In the present paper, we have described culture conditions that represent a significant improvement of the standard techniques for a rapid and reproducible expansion of osteogenic precursors from human bone marrow. In particular, we have shown the role of FGF-2 in maintaining osteogenic potential of BMSC during their *ex vivo* expansion.

The association of these cells with the appropriate bioceramics is a model for a potentiated guided regeneration of bone, where ceramic tridimensional scaffold provides the track for tissue regeneration, allows blood vessel invasion, and primes multicentric bone formation by BMSC. Such a biomaterial might be considered both osteoconductive and osteoinductive and possibly be used in human therapy as a high efficiency technique for large bone segment reconstruction.

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