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Short Communication

Selection and amplification of a bone marrow cell population and its induction to the chondro-osteogenic lineage by rhOP-1: an *in vitro* and *in vivo* study

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ABSTRACT The differentiation and maturation of osteoprogenitor cells into osteoblasts are processes which are thought to be modulated by transforming growth factors-beta (TGF-ß) as well as by bone morphogenetic proteins (BMPs). Osteogenic protein-1 (OP-1, also known as BMP-7) is a member of the BMP family, and it is considered to have important regulatory roles in skeletal embryogenesis and bone healing. Rat bone marrow cells were cultured *in vitro* in a collagen-gel medium containing 0.5% fetal bovine serum (FBS) for 10 days in the presence of 40 ng/ml recombinant human OP-1 (rhOP-1). Under these conditions, survival of the bone marrow cell population was dependent on the presence of rhOP-1. Subsequently, the selected cells were cultured for 6 days in medium containing 40 ng rhOP-1 and 10 % FBS. During the last 2 days, dexamethasone (10^{-8} M) and ß-glycerophosphate (2 mM) were added to potentiate osteoinduction. Concomitant with an up-regulation of cell proliferation, DNA synthesis levels, colony number and size were determined. Chondro-osteogenic differentiation *in vitro* was evaluated in terms of the expression of alkaline phosphatase, the production of osteocalcin and the formation of mineralized matrix. After culturing *in vitro*, cells were placed inside diffusion chambers or inactivated demineralized bone matrix (DBM) cylinders and implanted subdermically into the backs of old rats for 28 days. Biochemical, histological and immunocytochemical analyses provided evidence of cartilage and osteoid tissue inside the diffusion chambers, whereas bone was also observed inside the DBM implants. In conclusion, this experimental procedure is capable of selecting a cell population from bone marrow which, in the presence of rhOP-1, achieves skeletogenic potential under *in vitro* as well as *in vivo* environments.

KEY WORDS: *mesenchymal stem cells, osteoprogenitor cells, collagen matrix, BMPs, cartilage, bone*

Bone morphogenetic proteins (BMPs) are pleiotropic molecules which belong to the transforming growth factor-beta (TGF-ß) superfamily and are closely related in structure and function (Reddi, 1997). Several of these proteins have now been purified from bovine demineralized bone matrix (DBM), cloned and prepared as recombinant human proteins (Reddi, 1998). These proteins have been reported to induce the expression of bone matrix proteins and to play a regulatory role in the initiation of cartilage differentiation and the induction of bone-forming cells from pluripotent mesenchymal stem cells (MSCs; Lecanda *et al.,* 1997). These proteins have been reported to induce the expression of bone matrix proteins and to play a regulatory role in the initiation of cartilage differentiation and the induction of bone-forming cells from pluripotent mesenchymal stem cells (MSCs; Lecanda *et al.*, 1997). BMPs appear to be involved in skeletal embryogenesis and to be osteoinductive, both in *in vitro* and *in vivo* models (Welch *et al.,* 1998). BMP-2 or BMP-7 (also named "osteogenic protein 1" or OP-1) have been reported to enhance bone repair when applied either directly or combined with a carrier, in fracture healing (Bonn, 1999). OP-1 not only stimulates the synthesis of cartilage molecules in human articular chondrocytes cultured *in vitro* (Nishida *et al.*, 2000), but it also induces *in vivo* cartilage repair

Abbreviations used in this paper: AB, alcian blue; ALP, alkaline phosphatase; α-MEM, alpha-minimum essential medium; ß-GP, beta-glycerophosphate; BM, bone marrow; Ca, calcium; DBM, demineralized bone matrix; dex, dexamethasone; Dw, dry weight; FBS, fetal bovine serum; GAGs, glycosaminoglycans; HE, hematoxylin-eosin; MSCs, mesenchymal stem cells; OC, osteocalcin; PSH, picrosirius-hematoxylin; PSP, picrosirius polarization; PGs, proteoglycans; rhBMP, recombinant human bone morphogenetic protein; rhOP-1, recombinant human osteogenic protein-1; rhTGF-ß1, recombinant human transforming growth factor-beta1.

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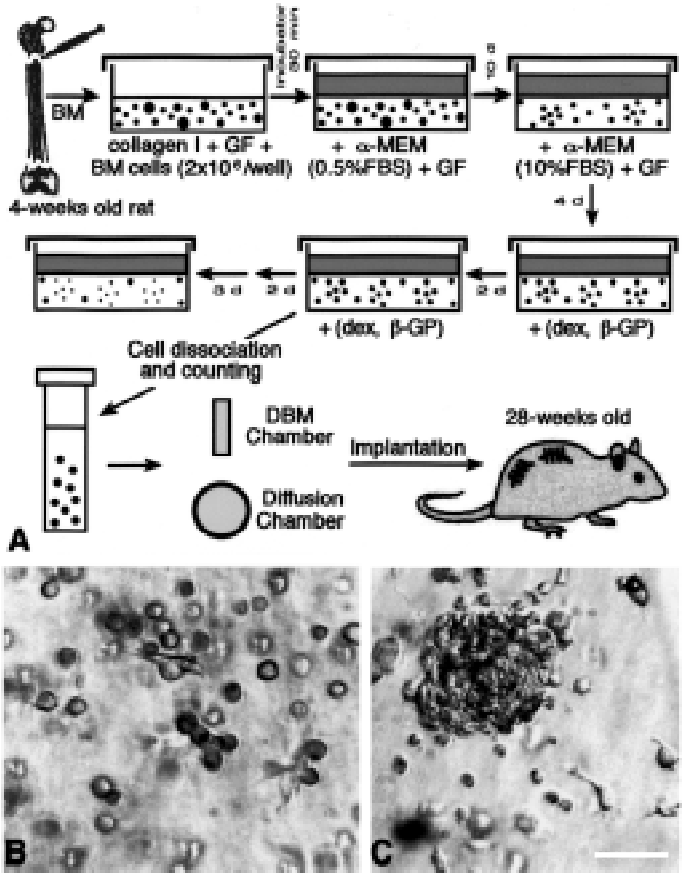

Fig. 1. Experimental procedure and appearance of cell cultures in collagen gels. (A) *For details of the experimental procedure, see Experimental Procedures. α–MEM, alpha-minimum essential medium; ß-GP, beta-glycerophosphate; BM, bone marrow; DBM, demineralized bone matrix; dex, dexamethasone; FBS, fetal bovine serum; GF, growth factor.* **(B)** *Cell cultures in collagen gel in the presence of rhOP-1 at the end of the selection period (day 10, round-shaped isolated cells), and (C) at day 18 where cells form conspicuous colonies. Bar, 100 μm for B and C.*

(Katic *et al.,* 2000). The first clinical reports on bone regeneration using recombinant human BMPs (rhBMPs) have been published only for BMP-2 and OP-1 (Geesink *et al.,* 1999). Although both BMPs are able to induce important beneficial effects in bone repair, significant differences can be observed in both circumstances (Groeneveld and Burger, 2000). In all cases, the targets of BMPs are mesenchymal cells, which are capable of being induced through the chondro-osteogenic lineage. These cells have been demonstrated to be derived from the MSCs of bone marrow, which significantly diminish in number with aging (Haynesworth *et al.,* 1994). We have recently developed an *in vitro* procedure which includes a progenitor cell compatible collagen gel, impregnated with different growth factors to select, amplify and induce a population of chondro-osteoprogenitor cells from rat bone marrow. These cells processed under the effects of a genetically engineered rhTGF-ß1, containing an auxiliary collagen binding domain (rhTGF-ß1-F2) (Andrades *et al.,* 1999; Becerra *et al.,* 2001), or BMP-2 (Andrades and Becerra, 2001) express skeletal markers and form hard tissue when they are implanted in animals. The present study evaluates the efficiency of rhOP-1 to select, amplify and induce a skeletogenic cell population from bone marrow, cultured in a collagen gel trap.

Cell number, expressed as DNA content (Table 1), suffered a decrease as a consequence of the starvation conditions associated with low serum concentration. After ten days of culture in collagen gels (Fig. 1B), the number of cells began to increase. Subsequently, cells cultured with rhOP-1 formed well-defined colonies (Fig. 1C), which increased in number and size. Mineralization of colonies was evident at day 14. Bone marrow cells cultured under control conditions never formed colonies and presented a morphology which was different to that observed when the cells were cultured in the presence of rhOP-1 (data not shown).

The expression of alkaline phosphatase (ALP) and osteocalcin, together with the deposition of calcium, which are all well-known markers of osteoblastic differentiation, were measured (Fig. 2). After the starvation period (from day 11 on), these values increased significantly in cultures which had been exposed to rhOP-1. Calcium deposition and osteocalcin levels in control conditions were imperceptible. These results show that rhOP-1 is mitogenic in this system, as has been shown for rhTGF-ß1-F2 (Andrades *et al.,* 1999) and rhBMP-2 (Andrades and Becerra, 2001). The formation of colonies and their mineralization is a characteristic of a certain differentiated state. The increase in expression of ALP and osteocalcin in cultures treated with rhOP-1 indicates that osteogenic induction has occurred, as has been claimed in other systems (Yamaguchi *et al.,* 1991). Therefore, on the basis of the differentiation criteria, it can be assumed that rhOP-1 selects *in vitro* a progenitor cell population from bone marrow, which can be directed along the osteogenic lineage under these experimental conditions.

The results of the biochemical analysis after *in vivo* implantation are summarized in Table 2. Histo- and immunocytochemical analyses facilitate an understanding of the distribution of collagen, proteoglycans (PGs) and other tissue components formed during the implantation period.

Sections of diffusion chambers containing non-treated culture cells showed a homogeneous appearance (Fig. 3A). In contrast, chambers filled with rhOP-1-treated cells presented a heterogeneous appearance, where conspicuous tissue condensations occupied an important volume of the implants (Fig. 3B). Parallel sections of these nodules showed that the regions with high alcianophilia (demonstrating the presence of sulfated glycosaminoglycans, GAGs), (box 1 in Fig. 3B), were not Gomori stained (Gomori stains mature bone matrix) (Fig. 3C), and were only weakly collagen II immunoreactive (Fig. 3D). These characteristics, together with other data regarding collagen I and IV (data not shown), indicate that this type of condensation presents a core of cartilage-like tissue, surrounded by a layer

TABLE 1

EFFECT OF EXPERIMENTAL CONDITIONS (CONTROL AND rhOP-1) ON DNA SYNTHESIS, COLONY NUMBER, MINERALIZED COLONY NUMBER AND COLONY SIZE DURING *IN VITRO* CULTURE IN THE PRESENCE OF rhOP-1

		Day				
		0	10	14	18	21
DNA (μg)	Control	22.50±3.2	2.28± 0.53	3.90± 0.41	5.20± 0.72	6.47± 0.68
	rhOP-1	22.50±3.2	3.28±0.31	6.20±0.52	8.25±1.63	12.32±2.54
Colony		ND	6 ± 0.8	11 ± 1.2	24 ± 2.3	28 ± 2.1
Mineralized colony		ND	ND	4 ± 0.2	11 ± 0.8	22 ± 1.1
Colony size (mm^2)		ND	0.7 ± 0.1	3.2 ± 0.2	3.8 ± 0.2	3.1 ± 0.3

ND, not- detected. No colony formation was detected under control conditions. Values are given as means ± SD for four samples.

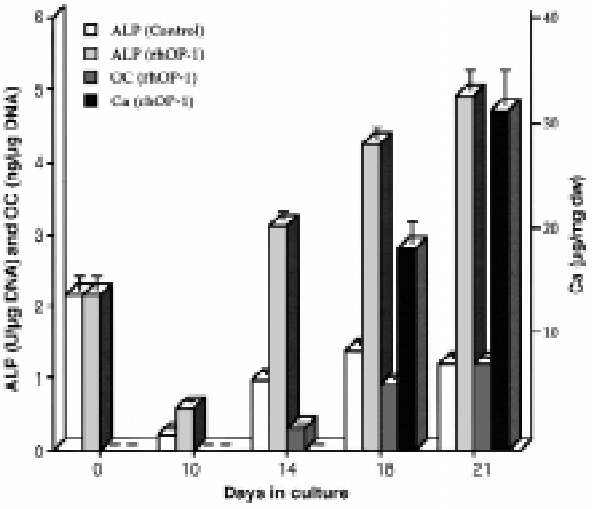

Fig. 2. Expression of alkaline phosphatase (ALP), osteocalcin (OC) and calcium (Ca) content in cell cultures at different times during the *in vitro* experiments. *Control cultures only express ALP. Cells in the presence of 40 ng/ml rhOP-1 progressively increased ALP expression after day 10, significantly above control levels. Ca deposition increased in cultures after a certain ALP activity (premineralization enzyme) had been reached. OC protein expression, which is indicative of mature osteoblast activity, was observed only during the final stages of culture.*

resembling osteoid-like tissue, as well as an outer perichondrium or periostium-like sheet, bordering the remaining fibrous tissue.

In contrast, alcian blue (AB)-negative condensations (Fig. 3B, box 2), were Gomori stained (Fig. 3E). Similar condensations near the wall of the chamber (Fig. 3B, box 3), presented a deep red color, which was more intense after papain digestion, when stained with picrosirius-hematoxylin (PSH), (Fig. 3F), probably reflecting immature bone matrix. In agreement with such a possibility, collagen I immunoreactivity was observed in these areas (Fig. 3G) while collagen II immunoreactivity was absent (data not shown).

Sections of DBM implants containing non-treated culture cells (controls; Fig. 3H) showed a better structured fibrillar tissue than controls in diffusion chambers. DBM implants with rhOP-1-treated cells were filled with a well-organized tissue with a consistent hard mass (Fig. 3I). A homogeneous tissue, abundant in ground substance and scattered isolated cells (light pink area), occupied a large part of the slide. Near the wall, a more cellular tissue with grouped cells could be seen (the wall is to the bottom in Fig. 3I). A tissue resolved in trabecular structures of dense collagenous tissue with individual cells in small lacunae occupied other extensive areas. These trabecula surrounded soft tissue with empty areas (upper part in Fig. 3I).

Under high magnification of the tissue near the wall, we observed conspicuous cells inside the lacunae, isolated or forming groups, consistent with an isogenic-like arrangement with intense birefringency with the picrosirius polarization (PSP) method (Fig. 3J). Positive Gomori staining was present in the extracellular matrix, and in regular bone matrix (Fig. 3K); weak collagen II immunoreactivity was observed in some cells of those areas (Fig. 3L). Light collagen I immunoreactivity was also observed in the cytoplasm of the cells (Fig. 3M). These characteristics of the tissue are indicative of a fibrocartilage-like structure.

The homogeneous tissue occupying the central areas (light pink area in Fig. 3I), presented thin collagen fibers in the ground substance among a few isolated cells. This ground substance did not present Gomori staining (Fig. 3K) or collagen II immunoreactivity (Fig. 3L), although anti-collagen I immunostaining could be observed (Fig. 3M). These characteristics are indicative of a cartilage-like tissue with some heterotypic components.

The above-described tissue appears to transform into a different one where bone characteristics emerged (Figs. 3I and N-P). Endochondral bone-like ossification events could be observed. Chondrocyte lacuna became wider and cavities with vascular-like elements could be seen (Fig. 3N). These cavities appeared to be enclosed by a new material forming trabecula densely stained with PSH, with some cells trapped on it, and rows of osteoblast-like cells lining the new material. Gomori staining (Fig. 3O) and collagen I immunoreactivity (Fig. 3P) were observed in those trabecula.

Our results using two types of implants (a closed system, diffusion chamber; and an open one, DBM chamber) clearly indicate that the process of histogenesis is strongly influenced by the environment in which cells are implanted. In diffusion chambers, cells treated with rhOP-1 form conspicuous condensations of a cartilage, osteoid and bone-like nature, whereas in DBM chambers, a more advanced and consolidated chondro-osteogenic tissue appears, in which the typical tissular quality of bone and cartilage can be observed.

Although the present results clearly demonstrate the cartilaginous and osseous identity of the tissue formed inside diffusion chambers, intramembranous or endochondral ossification could not be found in that circumstance. It is possible that an exact ossification process might occur inside the chambers without the presence of vascular elements. Our data show details that lead us to think that some special events take place in those situations. Moderate collagen IV and laminin immunoreactivity was observed in the presumed cartilage areas, whereas collagen II immunoreactivity was weak, even using previous enzymatic digestions to eliminate the ground substance usually masking collagen fibrils in cartilage. This may be explained by deviations of the differentiation pathway or by a transient transdifferentiation, as has been observed in other systems, particularly related to cell cultures (Hay, 1983). Similar results were obtained in these experimental systems when the implanted cells were treated with rhTGF-ß1-F2 (Andrades *et al.,* 1999; Becerra *et al.,* 2001) or rhBMP-2 (Andrades and Becerra, 2001).

We frequently observed cartilage nodules towards the center of the chambers and bone accumulation close to the filter, in agreement with other studies (Ashton *et al.,* 1980). This could be related to the nutritional situation of the different regions of the chambers. Thus, chondro- and osteogenesis could take place as independent

TABLE 2

BIOCHEMICAL ANALYSIS OF CHAMBER CONTENT AFTER IMPLANTATION UNDER CONTROL AND rhOP-1 – TREATED CONDITIONS

	Diffusion Chambers			DBM Chambers		
	ALP	OC	Ca	ALP	OC	Ca
Control	4.08 ± 0.7	3.2 ± 0.9	ND	20.0 ± 1.0	4.4 ± 0.8	6.0 ± 1.0
rhOP-1	12.65 ± 1.0	30.0 ± 2.0	2.54 ± 0.3	68.0 ± 2.1	32.7 ± 2.0	90.3 ± 5.7

Levels of ALP (U/µg DNA), OC expression (ng/µg DNA) and Ca content (µg/mg dw). ND, not-detected. Values are given as means ± SD for four samples.

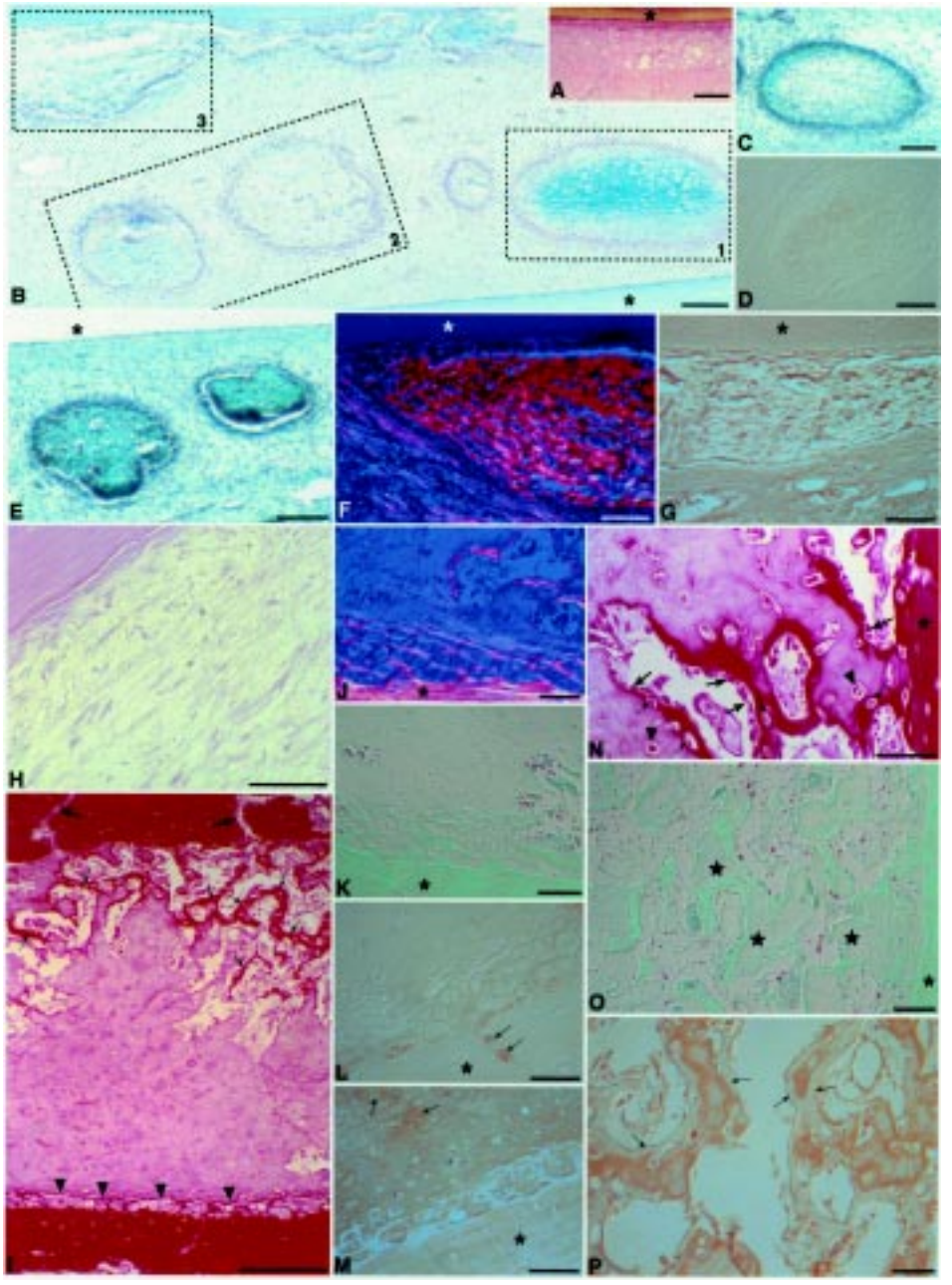

Fig. 3. Sections of diffusion chambers (A-G) or DBM chambers (H-P) after 28 days of *in vivo* implantation. (A) *A representative chamber with cells cultured under control conditions; the chamber is occupied by a fluid with some fibrillar tissue. Asterisk represents the wall of the chamber. PSH staining. Bar, 200 μm.* **(B)** *General view of the chamber content formed by cells pretreated with rhOP-1 in vitro. Distinct types of tissular condensations can be seen. Dotted boxes 1,2 and 3 are detailed in C,D,E and F with different staining techniques in contiguous sections. AB staining. Bar, 400 μm.* **(C)** *Condensation in B (box 1) stained with Gomori. Underneath the cellular sheet, a lightly stained area can be seen surrounding an unstained central core, which appeared stained in B.* **(D)** *A parallel section stained with anti-collagen II. A light net occupies the central AB stained area in B (box 1).* **(E)** *Tissular condensations in B (box 2) appeared stained with Gomori.* **(F)** *A condensation in B (box 3) processed with PSH stain and the PSP method. The deep red color indicates an immature bone-osteoid matrix near the wall of the chamber (asterisk).* **(G)** *A similar condensation presents collagen I immunoreactive fibrils. Bars, 500 μm.* **(H)** *Control DBM implants. The wall is sectioned in the top left corner. HE staining.* **(I)** *Tissue formed in a DBM implant containing cells pretreated with rhOP-1. Cartilage tissue with grouped cells (arrowhead), and trabecular tissue (small arrows) with cells placed in lacunae as bone tissue. New tissue penetrated inside the DBM matrix wall (arrows), where empty osteocyte lacunae can be observed. PSH staining. Bars, 500 μm.* **(J-M)** *Cartilage-like tissue formed in DBM implants in contiguous sections.* **(J)** *PSH staining of lacunar tissue, close to the wall (asterisk).* **(K)** *Parallel section stained with Gomori; the asterisk indicates the DBM wall.* **(L)** *Anti-collagen II immunoreactivity is limited to certain parts of the matrix of the lacunar area. Several cells have colonized the cavities of the DBM wall (asterisk) forming new matrices which are stained with this antiserum (arrows).* **(M)** *Anti-collagen I immunoreactivity in some cells of the lacunar area near the wall (asterisk), and also in the homogeneous tissue (arrows). Bars, 100 μm.* **(N-P)** *Trabecular bone-like tissue.* **(N)** *Osteoblast-like cells lining the new material everywhere (arrows); hypertrophic chondrocyte-like (arrowhead) and osteocyte-like cells inside the DBM wall (asterisk). PSH staining.* **(O)** *A contiguous section presents Gomori staining only in the new bone-like trabecula (asterisks).* **(P)** *A contiguous section illustrating collagen I immunoreactivity in the trabecula (arrows). Bars, 100 μm.*

processes in these circumstances from different cell lines or from the same cell line under different environmental conditions. Alternatively, transdifferentiation from chondrocytes to osteoblasts may occur, as has been suggested (Bianco *et al.*, 1998).

The mature nature of the tissue formed inside DBM implants is apparent. The morphological and chemical features of the tissue are much more like those of typical bone and cartilage, although a *sensu stricto* intramembranous or endochondral bone formation process is not easily identified.

Why did such a type of tissue appear inside DBM implants? Two cooperative circumstances could be implicated: i) the nature of the chamber walls, and ii) the violation of the implant contents by vasculature coming from outside. Cells implanted in DBM chambers could receive an extra dosage of a variety of bioagents (probably BMPs) coming through the wall of the implant or perhaps its collagen scaffold could exert a chemotactic action on BMPs issuing from the host tissular environment. This idea must imply a synergy or cooperative relationship between OP-1 *in vitro* applied to the cells, and BMPs (we don't know which one) coming from the host and targeted to the implanted cells. Such a type of synergy has been claimed by Si *et al.* (1998) between TGF-ßs and BMP-2, and among BMP-2, -3 and -7 (Thomadakis *et al.*, 1999).

Regarding vascular ingrowth, the porosity of the DBM wall allows capillary infiltration and endothelial cell contribution to vessel formation and consequently, enhancement of bone formation, as was shown by Villanueva and Nimni (1990). Obviously, the porosity of this type of open implant raises the question about the origin (donor or host) of the cells which are responsible for the tissue formed inside. However, neither bone nor cartilage was found when DBM implants were filled with cells pretreated with bFGF (Andrades *et al.,* 1999) or without treatment (control). Since no markers for stem cells have been used to distinguish host from donor cells, indirect proof can only be claimed: i) the procedure of bone marrow extraction ensures that no bone cell contamination exists in cultures; ii) starvation and amplification during *in vitro* periods determine that only undifferentiated cells, such as stem cells, could remain after that culture time and iii) bone marrow cells cultured under control conditions (without OP-1 treatment) did not induce any skeletogenic effect. It is conceivable that OP-1 added to the cultures might attract stem cells from the host. However, the half-life of this factor is probably incompatible with that possibility, because OP-1 was added more than two days before implantation, besides washing, isolation and the general manipulation of cells, prior to implantation. Overall, our results indicate that the implanted donor-derived cells are responsible for the DBM implant skeletogenesis.

Experimental Procedures

Isolation of bone marrow stromal cells and collagen gel preparations were performed as described elsewhere (Andrades *et al.,* 1999). Culture preparations contained 150 µl of collagen (0.35 mg/ml) mixed with 40 ng/ml rhOP-1 (Creative Biomolecules). Cells were mixed with the collagen-rhOP-1 solution at a density of $2x10^6$ cells/150 µl collagen/well in 48-well plates. The culture plates, 6 plates per experiment, were left 30 min at 37°C to allow the collagen to gel. Then, 150 µl/well of α-MEM containing rhOP-1 were added on top of the collagen gel and cultures were maintained in standard conditions. The medium was changed every 3 days and fresh rhOP-1 was added, according to the diagram shown in Fig. 1A. Control cultures did not contain rhOP-1. The culture medium consisted of α-MEM containing 100 µg/ml Penicillin G (Sigma Chemical Co.), 50 µg/ml Gentamicine Sulfate (Sigma) and 0.3 µg/ml Fungizone (GIBCO). This medium contained 0.5% FBS for the initial 10 days of culture, and 10% FBS up to the end of the *in vitro* experiment. Dexamethasone (10^{-8} M, Sigma) and ß-glycerophosphate (2 mM, Sigma) were added to the cultures to facilitate the induction of osteogenesis.

Biochemical parameters were measured as reported elsewhere (Andrades *et al.,* 1999). After 16 days of *in vitro* culture, collagen gels were digested with collagenase (0.05% collagenase type I, Worthington Biochemical Corp.) as previously described (Andrades *et al.,* 1999), and cells were dissociated, centrifuged, washed, resuspended, counted and transferred to DBM and diffusion chambers. In this study, a total of 130 DBM implants (10 mm diameter x 2mm), and 70 diffusion chambers (14 mm diameter x 2 mm; Millipore Ltd.), were implanted in 20 animals. In each chamber, $5x10^5$ cells processed *in vitro* were implanted. Twenty, eight-week-old male isogenic (inbred strain) Fischer 344 rats were anesthetized and surgery was performed as described (Andrades *et al.,* 1999). Four weeks later the implants were harvested.

All procedures were carried out at room temperature. Implants were fixed and decalcified; the plastic ring of the diffusion chambers was dissolved in toluene. Dehydration, paraffin embedding, cutting and staining with alcian blue, picrosirius-hematoxylin and hematoxylin-eosin were routinely performed. When necessary, digestions on tissue slides were performed with papain (Papain IV, Difco Laboratories), hyaluronidase (testicular hyaluronidase, Sigma) or chondroitinase AC (Miles Laboratories).

Immunocytochemistry was carried out using specific antisera to collagen I, II, III, IV and to laminin (Chemicon International Inc.). To test the antisera, different rat tissues were used as controls. Images were obtained with a Zeiss III (Zeiss) inverted phase contrast microscope and a Nikon Microphot FXA (Nikon).

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