

Different response to osteo-inductive agents in bone marrow- and periosteum-derived cell preparations

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Rabbit bone marrow- and periosteum-derived cells were cultured in medium containing dexamethasone, bone morphogenetic protein-2 (BMP2) or both. The response of bone marrow-derived cells, measured as alkaline phosphatase expression, depended on the stage of growth. In subconfluent cultures, BMP2 had a greater effect than dexamethasone and treatment with both further increased enzyme activity. In confluent cultures, the effect of dexamethasone was greater than that of BMP2 and, when used together, they had an additive effect. The mineral deposition observed in these cultures did

not have the typical structure of bone nodules. For periosteum-derived cells, dexamethasone did not increase the expression of alkaline phosphatase, while BMP2 did; treatment with both was less effective than treatment with BMP2 alone. Typical bone nodules were observed in cultures of periosteum-derived cells treated with dexamethasone and BMP2. These findings indicate that either osteoprogenitor cells from these two sources are intrinsically different or else non-progenitor cells in the preparations directly or indirectly affect the responsiveness to osteo-inductive agents.

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Although both bone marrow and periosteum are involved in the development, remodeling and repair of bone and have been thought to contain osteochondral progenitor cells (Fell 1925, Owen and Friedenstein 1988, Caplan 1994), their connective tissue scaffoldings are different. Moreover, the bone marrow scaffolding, called stroma, provides unique and specific multifactorial support for hematopoiesis as well as housing for osteochondral progenitor cells.

Cell preparations from these two tissues have been used to study chondrogenesis and osteogenesis both *in vivo* (Nakahara et al. 1990, Wakitani et al. 1994) and *in vitro* (Nakahara et al. 1991, O'Driscoll et al. 1994). These cell preparations have also been used in models of bone and cartilage repair/regeneration. Cell preparations from both bone marrow (Ashton et al. 1980, Jaiswal et al. 1997) and periosteum (Nakahara et al. 1990, 1991) have been shown to contain osteochondro-progenitor cells that can be isolated and expanded in culture. Periosteum-derived cell preparations from different sites have been compared in a chondrogenesis model (Gallay et al. 1994), but bone marrow- and periosteum-derived cell preparations from the same animal have not been directly compared in parallel assays.

We compared these two preparations side by side in an assay for their osteogenic potential by culturing them in an osteo-inductive medium containing dexamethasone, BMP2, ascorbate-2 phosphate and β -glycerophosphate. Such a medium appears to drive osteoprogenitor cells into the osteogenic lineage, which results in the formation of mineralized matrix in 2–3 weeks (Tenenbaum and Heersche 1985, Bellows et al. 1986, Maniatopoulos et al. 1988, Rickard et al. 1994). Our hypothesis is that the cells from these two sources are intrinsically different.

Material and methods

Cells were harvested from 11 2-month-old rabbits and, in each case, bone marrow and periosteum were compared.

Cell culture

Bone marrow-derived cells. Under anesthesia, the proximal medial surface of the tibia was exposed through a small incision. After removal of the periosteum, as described below, the tibia was perforated with a 16-gauge needle. Bone marrow (4–5 mL) was

then aspirated from the tibial shaft through a preheparinized plastic tube, affixed to a 10-mL syringe containing 1 mL of heparin solution (3,000 units/mL). Under sterile conditions, the aspirate was transferred into a 50-mL plastic tube and centrifuged at 1,000 g for 10 minutes. The pelleted cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Laboratories, Gaithersburg, MD, USA) supplemented with 1% antibiotic-antimycotic solution (10,000 units of penicillin G, 10 mg of streptomycin and 25 µg of amphotericin B/mL) and with 10% fetal bovine serum (FBS; Intergen® Company, Purchase, NY, USA) from a selected lot (Lennon et al. 1996). An aliquot of this cell suspension was combined with an equal volume of 4% acetic acid to lyse the red blood cells prior to cell counting with a hemocytometer. The cell suspension was then seeded into sixteen 21-cm² culture dishes at a starting density of 3.6×10^5 nucleated cells per cm².

Periosteum-derived cells. The skin and soft tissue were gently retracted to expose the periosteum. A 1-cm × 2-cm piece of periosteum was first cut with a scalpel blade and then removed from the bone with a pair of forceps. The pieces of periosteum obtained from both tibiae were put into a 50-ml plastic tube containing Tyrode's balanced salt solution (T2145; Sigma, St. Louis, MO, USA) and transferred to the culture room. Under sterile conditions, the periosteal fragments were washed twice with Tyrode's solution and then minced into small pieces with two scalpel blades. The minced periosteum was then put into a 50-mL plastic tube containing 2 mL of 0.5% collagenase (CLSII; Worthington Biochemical Co., NJ, USA; 189 units/mg) in DMEM and incubated for 2 hours at 37 °C with vortexing for 10 seconds every 15 minutes. The digestion was stopped by adding an equal volume of calf serum (Hyclone Laboratories, Inc., Logan, UT, USA). The cells were pelleted by centrifugation at 1,000 g for 10 minutes and the pellet was resuspended in DMEM, supplemented with 1% antibiotic-antimycotic solution and with 10% FBS (Intergen) from a selected lot (Lennon et al. 1996). An aliquot of the cell suspension was used for cell counting with a hemocytometer. The cells were then plated into 16 21-cm² culture dishes at a starting density of 10^5 cells per cm².

Culture conditions. The culture dishes were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The first change of medium performed at 3 days removed most nonadherent cells. At this first change of medium and at every change of medium thereafter, the medium for a group of 4 plates (control) was supplemented with 50 µM ascorbate-2 phosphate, the medium for a second

group (Dex) was supplemented with 50 µM ascorbate-2 phosphate and 10^{-7} M dexamethasone, the medium for a third group (BMP2) contained 50 µM ascorbate-2 phosphate and 30 ng/mL BMP2 (human recombinant bone morphogenetic protein-2, generously supplied by the Genetics Institute, Cambridge, MA, USA) and the medium for the fourth group (Dex+BMP2) was supplemented with 50 µM ascorbate-2 phosphate, 10^{-7} M dexamethasone and 30 ng/mL BMP2. On day 9 and at every change of medium thereafter, the medium for each group was also supplemented with 10 mM β-glycerophosphate. It was changed every 3 days.

Analysis of the cultures

Quantitative measurement of alkaline phosphatase activity. Since alkaline phosphatase is a cell-surface enzyme, alkaline phosphatase activity was measured in living cultures. The medium was withdrawn and the cells were washed twice with 5 mL of Tyrode's balanced salt solution. A 2-mL aliquot of alkaline phosphatase substrate buffer (50 mM glycine, 1 mM MgCl₂, pH 10.5), containing the soluble chromogenic alkaline phosphatase substrate (2.5 mM p-nitrophenyl phosphate), was added directly to the dish at room temperature. During incubation, cell-surface alkaline phosphatase converts p-nitrophenyl phosphate into p-nitrophenol, which then takes on a yellow color. 15 minutes after substrate addition, 1 mL of the buffer was removed from the culture and mixed with 1 mL of 1 N NaOH to stop the reaction. The absorbance of the mixture was read in triplicate on an ELISA plate-reader (Bio-Rad) at 405 nm. Due to high inter-individual variability, the alkaline phosphatase activity values were standardized as a function of matching control values.

Alkaline phosphatase histochemistry. Since the adherence and morphology of the cells were maintained over the 15 minutes of the alkaline phosphatase assay, the same cells were washed twice with 5 mL of Tyrode's solution and then histochemically stained for alkaline phosphatase with a kit from Sigma Diagnostics (Kit # 85), following the procedure recommended by the manufacturer. Briefly, cells were fixed in acetone-citrate buffer for 30 seconds and washed twice with double distilled water. A 2-mL aliquot of the substrate solution (Fast violet B salt, Naphtol AS-MX) was then added to each dish and the plates were incubated in the dark for 45 minutes at room temperature. The reaction was stopped by removing the substrate solution and washing twice with 5 mL of distilled water. Cells expressing alkaline phosphatase on their surface developed a red stain after the incubation period.

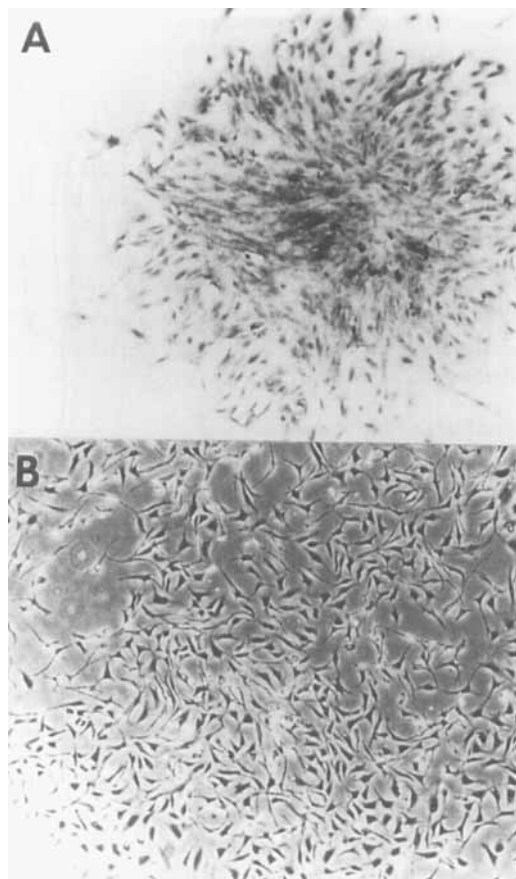


Figure 1. Microscopic appearance of the cultures. Phase contrast photomicrograph of bone marrow-derived cells (A) and periosteum-derived cells (B) on day 6 of culture, $\times 40$.

von Kossa staining. Dishes stained for alkaline phosphatase were rinsed twice with 5 mL of distilled water. A 2-mL aliquot of freshly prepared 2% (w:v) silver nitrate in water was added to each dish and the dishes were kept in the dark for 10 minutes. The plates were rinsed again with 5 mL of distilled water and exposed to bright light for 15 min, with this layer of water covering the cells. The reaction was stopped by rinsing thoroughly with distilled water. The presence of mineral deposition was indicated by the development of a black precipitate on the mineralized matrix.

Statistics

11 individual 2-month-old rabbits were harvested, and, in each case, bone marrow and periosteum from the same rabbit were compared; duplicate cultures of each one of the groups of the study were analysed at each time-point. Since the alkaline phosphatase activity data failed to pass normality and/or equal variance

tests, a Friedman repeated measurements anova on ranks was run for each cell type at each time-point. To isolate the group or groups that differ from the others, a Student-Newman-Keuls all pairwise multiple comparison test was used within each cell type and time-point. P-values < 0.05 were considered significant.

Results

In all groups, the cells from bone marrow aspirates initially formed individual colonies of fibroblastic cells that could be observed macroscopically on day 6; the colonies increased in size and reached confluence in 12–16 days. The rate of proliferation and the time at which confluence was achieved varied between individual rabbits. In the periosteum-derived preparations, the cells never formed colonies (Figure 1); rather they were uniformly distributed over the culture surface, divided rapidly and reached confluence at approximately day 7 of the culture, 5–7 days earlier than the bone marrow-derived cultures. The time at which confluence was reached by the periosteal cells was less variable between individual rabbits than in the bone marrow-derived cells. Even when seeded at 0.5 or 0.25×10^5 cells per cm^2 , the periosteal cell cultures grew rapidly and reached confluence no later than day 8.

The response of bone marrow-derived cells to the added factors, measured as expression of alkaline phosphatase, depends on the stage of growth of the cells (Table). In subconfluent cultures (day 11), the alkaline phosphatase values of the dexamethasone-treated group were higher than those of the control group. The BMP2-induced increase in alkaline phosphatase activity was higher than that observed with the addition of dexamethasone alone, and treatment with both substances further increased the enzyme activity. When cells were confluent (day 18), the effect of dexamethasone was greater than that of BMP2, and when both were present, the level of expression of alkaline phosphatase was even greater.

In contrast to the bone marrow cells, the periosteum-derived cells, with respect to alkaline phosphatase activity, showed the same pattern of responses to the different treatments on both day 11 and day 18. In other words, for periosteal cells, BMP2 gave the greatest response in alkaline phosphatase activity at both time-points (Table), while, for bone marrow, BMP2 gave a greater response than dexamethasone on day 11 and dexamethasone gave a greater response than BMP2 on day 18 (Table). However, at both time-points, the periosteal cultures were confluent, which may have influenced the responsiveness of the cells.

Alkaline phosphatase activity values (median range) of the experimental groups relative to controls in bone marrow (B.m.)- and periosteum (P.)-derived cells on days 11 and 18 of culture. The value of alkaline phosphatase activity of the control groups was set to 1 for each rabbit

	Dex		BMP2		Dex+BMP2	
B.m. d11	a,c,d 2.24	0.53- 5.14	a,b,d 3.82	1.62-7.95	a,b,c 8.42	1.83-10.0
B.m. d18	a,c,d 7.81	3.96-19.0	a,b,d 3.81	0.87-10.0	a,b,c 9.15	3.32-28.0
P. d11	a,c,d 1.76	0.33- 2.11	a,b,d 11.5	1.84-21.5	a,b,c 4.22	2.00-15.2
P. d18	c,d 1.87	0.36- 5.80	a,b,d 19.8	1.84-34.6	a,b,c 7.26	0.56-13.5

^a different from Control at $p < 0.05$

^b different from Dex at $p < 0.05$

^c different from BMP2 at $p < 0.05$

^d different from Dex+BMP2 at $p < 0.05$

In periosteal cultures, BMP2 supplementation was the most effective treatment for increasing enzyme activity. Dexamethasone had no dramatic effect by itself and, in combination with BMP2, it attenuated the effect of BMP2. The alkaline phosphatase values of the control group and the dexamethasone-treated group on day 18 were the only two groups that did not differ in all the paired comparisons

No mineral deposition was observed in any culture on day 11. By day 18, no such deposition was revealed by von Kossa staining in untreated or BMP2-treated cultures of either type of cells or in periosteal cells treated with dexamethasone alone. In the dishes

that stained positively for mineral deposition (Figure 2), two different types of mineralized matrices were observed. In the first type, mineralization was spread over large areas throughout a monolayer of alkaline phosphatase-positive cells. The size of these areas varied. This type of mineralization was observed only in bone marrow-derived cells treated with dexamethasone alone or in combination with BMP2. In the second type, mineralization was restricted to well defined, three dimensional structures, termed nodules. The nodules exhibited intense alkaline phosphatase activity and were scattered on top of a confluent monolayer of alkaline phosphatase-negative cells

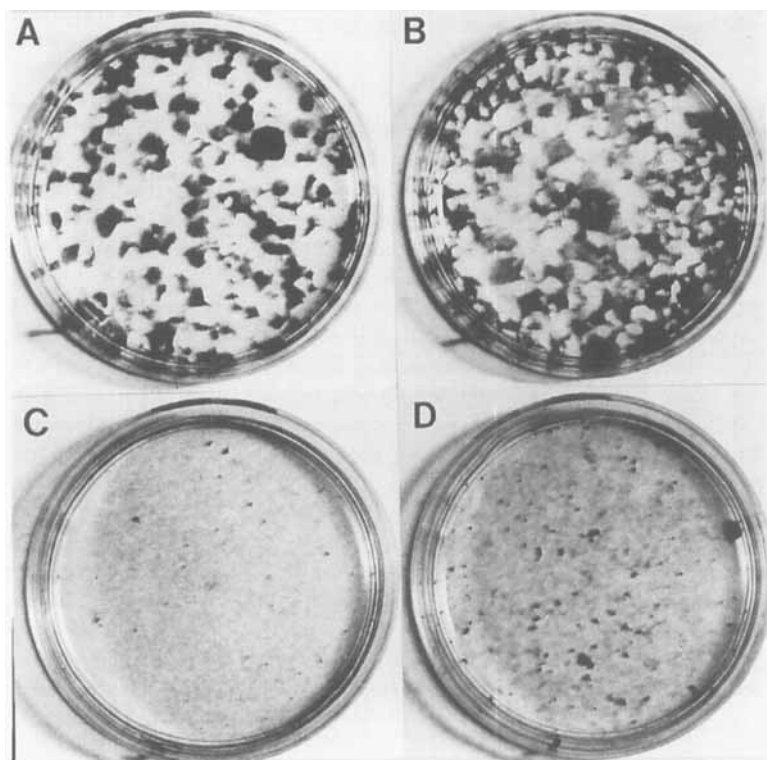


Figure 2. Primary cultures of bone marrow- and periosteum-derived cells.

Macroscopic aspect of the culture dishes (21 cm²) after double staining for alkaline phosphatase histochemistry and mineral deposition (von Kossa).

A. Bone marrow-derived cells treated with dexamethasone

B. Bone marrow-derived cells treated with dexamethasone and BMP2.

C. Periosteum-derived cells treated with dexamethasone.

D. Periosteum-derived cells treated with dexamethasone and BMP2.

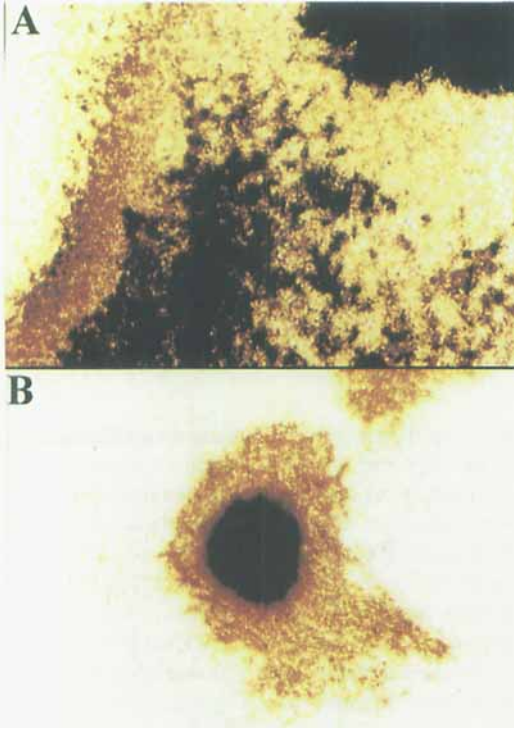


Figure 3. Histologic appearance of the mineralized matrix of the cultures treated with dexamethasone and BMP2. Microscopic appearance of the culture dishes after double staining for alkaline phosphatase histochemistry (red) and mineral deposition by von Kossa staining (black). Bone marrow-derived cells (A) and periosteum-derived cells (B) on day 18 of culture, $\times 20$.

(Figure 3). This bone nodule-associated mineralization was observed in the periosteum-derived cells treated with dexamethasone and BMP2. Similar mineralized bone nodules have been observed in subcultured rat bone marrow stroma cell cultures (Maniopoulos et al. 1988), but their formation was dependent only upon the presence of dex. Such bone nodule formation from periosteum-derived cells has not been documented before. Smaller, non-mineralized nodules were visible in periosteum-derived cells treated with dexamethasone alone. Periosteum-derived cells, untreated or treated with BMP2 alone, did not form nodules.

In a separate series of experiments, periosteum-derived cells were seeded at lower densities in the same experimental groups. In those cases, the number of alkaline phosphatase-positive cells or groups of such cells in the Dex and Dex+BMP2 groups was decreased and became undetectable when cells were seeded at fewer than 0.5×10^5 cells per cm^2 .

Discussion

The cells present in two preparations of osteoprogenitor cells show a different behavior when introduced into culture. Bone marrow-derived preparations are a complex mixture of different cell types, including stroma-derived cells and cells from the hemopoietic lineage. The culture conditions employed here select for a small subpopulation of culture-adherent cells, mainly mesenchymal progenitor cells, which exhibit a fibroblastic morphology on the plates and proliferate to form colonies, visible as of day 6. Periosteum-derived preparations are intrinsically less complex and include only two prominent cell populations derived from the cambium layer or the fibrous layer. These cells adhere to the plate and divide so rapidly that individual colonies cannot be distinguished on the plates. The differences between these two cell preparations are more obvious when expression of alkaline phosphatase is measured. Both dexamethasone and BMP2 can increase the expression of this enzyme by bone marrow-derived cells, whereas only BMP2 can do so with periosteum-derived cells.

The expression of alkaline phosphatase in response to the treatments depends on the stage of growth of the bone marrow-derived cells. In subconfluent cultures, with the cells actively proliferating, alkaline phosphatase activity is higher in the BMP2-treated than in the dexamethasone-treated group. In confluent cultures, when the rate of proliferation of the cells has slowed, dexamethasone-treated cells have a greater alkaline phosphatase activity than BMP2-treated cells. These results may indicate that once these cells have reached confluence, they are more sensitive to osteo-inductive treatments and that at the concentration used in this study, BMP2, on its own, is not a suitable osteo-inductive agent to promote the differentiation of these progenitor cells. BMP2 has been reported to induce osteoblastic phenotypes in murine stromal cell lines (Katagiri et al. 1990, Ties et al. 1992, Beresford et al. 1993, Wang et al. 1993, Dennis and Caplan 1996). Although BMP2 is not effective in inducing bone nodule formation in rat bone marrow-derived cells, it can accelerate and enhance the dexamethasone-induced osteoblastic differentiation process (Beresford et al. 1993, Rickard et al. 1994). No comparable data are available for rabbit cells.

As regards periosteum-derived cells, no differences were observed between the two time-points examined. These cells were confluent on day 7 and the rate of proliferation seemed not to be affected by confluence. Only treatment with BMP2 alone seemed to be effective in increasing alkaline phosphatase activity in these cultures but, as with the bone marrow-de-

rived cells, this treatment was not sufficient to promote terminal differentiation of the progenitor cells along the osteogenic pathway. The combination of dexamethasone and BMP2 was the only treatment that led to mineralized matrix during the study.

From the results presented above, we suspect that the main difference between these two preparations of cells resides in the number and rate of proliferation of "contaminant" non-progenitor cells. In the case of periosteal preparations, this non-progenitor population probably represents a high percentage of the cells in the cultures and, although these cells can express alkaline phosphatase when exposed to BMP2, they cannot differentiate along the osteogenic pathway and form mineralized matrix, as suggested by the series of experiments in which a dilution of the cells led to a decrease in the number of alkaline phosphatase- and von Kossa-positive nodules. Only a few cells in these periosteal preparations have osteogenic potential when exposed to dexamethasone and BMP2. Unfortunately, there are no specific markers to distinguish the progenitor cells from cells lacking osteogenic potential. Therefore, one cannot calculate the proportions in which these two subpopulations of cells are present accurately or purify the progenitor cells from the "contaminant" non-progenitor cells.

In bone marrow-derived cultures, the proportion of osteoprogenitor cells among the culture-adherent cells seems to be higher, since the amount of mineralized matrix formed in these cultures was much greater than in the periosteum-derived cultures. Nevertheless, the relative number of progenitor cells in these preparations may not be the only difference; the type of mineralization and its localization were also different. In bone marrow cultures treated with dexamethasone alone or in combination with BMP2, the mineralization process started at the sites where two colonies merged and then rapidly spread onto the neighboring matrix. The heterogeneity intrinsic to the bone marrow preparations seems to provide more and/or better mineralization foci which allows a combination of dystrophic and skeletal mineralization. A similar type of mineralization in primary cultures of adherent cells from rabbit marrow has been reported by Howlett et al (1986). In periosteum-derived cultures treated with dexamethasone and BMP2, the alkaline phosphatase-positive cells appeared in small clusters on top of alkaline phosphatase-negative cells and, eventually, these groups of cells became mineralized without spreading onto other areas.

Both populations of cells used in this study contain osteoprogenitor cells capable of differentiation into the osteogenic lineage and of forming mineralized matrix *in vitro*, under the appropriate culture condi-

tions. The results presented above indicate that the preparations obtained from these two tissues are not equivalent. They show differences in the pattern of growth, their response to inductive factors and the type of mineralized matrix which they form under these conditions. We believe that these differences may be due to the presence of various types of cells in various proportions, modulating or masking the response of the progenitor cells in these preparations. When performing *in vitro* experiments with preparations of cells from these two osteochondrogenic tissues, caution should be used when interpreting the results, since we currently have no criteria for judging the purity of the cell preparations. Obviously, the ratio of progenitors to non-progenitors can dictate the level of response to inductive agents.

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