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Mineralization and Alkaline Phosphatase Activity in Collagen Lattices Populated by Human Osteoblasts

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Abstract. Adult human osteoblastic cells were grown in a native type I collagen gel. Proliferation and viability analyses showed that cells rapidly stopped dividing and became blocked in the G0G1 phase (91% on day 13). Carboxyfluorescein diacetate cell staining and flow cytometry showed that osteoblasts were viable for the first 16 days and then viability decreased (58% viable cells on day 22). Osteoblasts were able to retract the matrix. Betaglycerophosphate (βGP) stimulated the deposition of mineral particles in the collagen network, and electron probe microanalysis showed that they were principally calcium and phosphorus, with a Ca/P ratio of about 1.7. Various times of βGP supply were tested. We compared 10 mM βGP added only once at day 0, or continuously from day 0, day 8, or day 21. Mineralization was observed in conditions where βGP was added at day 0. Furthermore, 10 mM βGP added once during gel preparation was sufficient to induce mineralization with mineral accumulation up to day 15 whereas the speed of the gel contraction decreased. In every condition, cultures expressed high alkaline phosphatase (ALP) levels as early as day 3, which decreased afterwards. These kinetics might explain why the other conditions did not prove favorable to the mineralization process. The model was used to study the influence of blocking gel retraction. Blocking retraction delayed the ALP activity decrease, but had no effect on mineralization. In conclusion, human adult osteoblasts cultured in native collagen gel stopped proliferation and underwent mineralization very early. This model should be used to investigate the influence of effectors on the early stages of culture.

Key words: Osteoblast — 3-Dimensional culture — Mineralization — Alkaline phosphatase — Collagen gel.

Bone cells produce organic matrix that mineralizes to form calcified tissue. Most *in vitro* studies of mineralization used monolayer cultures of osteoblastic murine [1–3] or avian origin [4] cells, cell lines established from osteosarcomas [5–7], or normal human cells [8, 9]. Cells were cultured until they formed multilayers with nodules [10]. Mineralization was observed only after the nodule formation occurred. Phosphate, provided by β-glycerophosphate (βGP) was used routinely in most of the studies on bone cell mineralization in culture. Supplementation of culture medium with βGP induced osteogenesis and promoted hydroxyapatite deposition around osteoblasts [1, 11]. The role of alkaline phosphatase (ALP) during the mineralization process, as well as the implication of βGP only as a phosphate source or as a modulator of bone cell metabolic activity is not clearly established [12, 13]. Using monolayer cultures, Tenenbaum [14] reported inhibition of ALP activity, whereas Chung et al. [15] found that βGP had no effect on ALP.

The aim of this study was to test a 3D system in order to assess whether the matrix maturation phase, well known in 2D conditions [10, 11], is needed before initiation of the mineralization process. The present study uses an *in vitro* system that closely mimics a tissue-like environment with a three-dimensional culture in a type I collagen gel. Adult human osteoblastic cells that retained their osteoblastic phenotype during the first few passages were used [16]. We first characterized cell proliferation pattern and viability within collagen gels as well as cell ability to retract the matrix. This ability was well described for fibroblastic cells [17] and for other osteoblastic cell types [18]. Then we examined the effect of various timings in βGP administration on the mineralization process and how this mineralization process was modulated in free-floating gels as compared with nonretracting gels.

Material and Methods

Cell Culture

Bone fragments were taken during surgical hip replacement from subjects aged 50–80 years and digested with trypsin and collagenase [16]. Cells collected from the second and the third digestions were grown in Earle's Minimum Essential Medium/MEM-Ham F12 (1:1) supplemented with 1 mM pyruvate, 2 mM glutamine, 50 μg/ml ascorbic acid, 5 μM non-essential aminoacids, 50 μg/ml penicillin and streptomycin, and 2% Ultroser (Gibco BRL). Cells were used between the second and sixth passages.

Human skin was obtained by surgical excision from young patients undergoing plastic surgery of the ears. Fibroblasts from explants were propagated in serial subcultures. The cells were grown in MEM supplemented with 10% calf serum (CS).

Collagen Lattice Preparation

Gels were prepared according to Bell et al. [17] using type I acid

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soluble calf skin collagen purified as previously described [19]. This preparation was hexosamine free, contained less than 3% type III collagen, and type I collagen contained telopeptides. Lattices were prepared in 60 mm bacteriological Petri dishes by rapidly mixing 2×10^5 cells with 1.5 ml collagen (2.4 mg/ml in 0.1% acetic acid) in a final volume of 5 ml using 1.76 × concentrated medium. Experiments were carried out in MEM supplemented with 10% new-born CS. Gels contained 10 mM βGP, except where otherwise stated. The culture medium was generally changed after 8 days, at the end of retraction. Fixed gels were made by placing an o-ring of scrynel nylon (3 mm wide) at the bottom of a Petri dish then pouring the gel mixture into the dish [20], so that the gel did not contract.

Viability of Osteoblasts Cultivated in Lattice

Cell viability was checked using carboxyfluorescein diacetate (CFDA, Molecular Probes, Eugene, OR), which passively crosses cell membranes and is converted to a polar fluorescent product by intracellular esterases. Cells were released from the gel by collagenase digestion: lattices were rinsed three times with PBS, digested with 1 mg/ml collagenase Sigma type V at 37°C for up to 30 minutes, depending on the age of the lattice. They were collected by centrifugation and rinsed with phosphate-buffered saline (PBS). 2×10^5 cells were suspended in 200 μl PBS and incubated with 30 μg CDFA for 30 minutes at 37°C, 5% CO_2. The CFDA was prepared fresh from a stock solution (20 mg/ml in acetone) kept at −20°C. The cells were rinsed twice with PBS, suspended in 200 μl PBS, and analyzed by flow cytometry in a Facstar Plus cell sorter (Becton Dickinson) equipped with an argon laser emitting 250 mW excitation at 488 nm. Five thousand cells were analyzed in each sample. Specific green fluorescence due to fluorescein was analyzed through a 530 ± 40 nm bandpass filter.

Cell Cycle and DNA Assay

Cells were released from the gel by collagenase digestion, as described for studies of viability. For cell cycle analysis, cells were permeabilized with 70% ethanol at +4°C for 30 minutes, then pelleted by centrifugation and incubated at 37°C for 10 minutes with 25 μl ribonuclease A (1000 IU/ml) per 25×10^4 cells. PBS (100 μl) was added and the cells were stained by adding propidium iodide (PI) to a final concentration of 50 μg/ml. Flow cytometry analysis was performed with a Facstar Plus Cell sorter (Becton Dickinson). Gating was performed using width-versus-area dot plots to exclude doublets and multiplets. Data were processed using CellFit software. A total of 10,000 cells were acquired for each sample. Red fluorescence signals due to PI emission were collected through a 585/40 nm bandpass filter. DNA was assayed with bisbenzimide [21].

Determination of ALP

Cells were released from the gel by collagenase digestion as described above. They were pelleted by centrifugation, suspended in 0.5 ml 50 mM Tris, 0.15 M NaCl, pH 7.4, and sonicated at +4°C. The lysate was centrifuged for 10 minutes at 2500 rpm, and 20 μl of supernatant was used to assay ALP using a p-nitrophenylphosphate substrate (Merck n° 12950). In some experiments, cells grown in monolayers in MEM-Ham F12-Ultroser medium were used as controls. Cells were harvested by trypsinization, pelleted by centrifugation, then treated as the cells collected from the gel. Protein in the supernatant was measured by the method of Lowry et al. [22].

Mineralization Analyses

Von Kossa staining was carried out after 2–4 weeks in culture. The lattices were fixed by immersion in 10% formalin in PBS for 4 hours, processed, and embedded in paraffin: sections were cut (5 μm) and stained by the Von Kossa technique.

Transmission electron microscopy (TEM) and energy-dispersive X-ray microanalysis were done on lattices rinsed in PBS, fixed with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, postfixed with 1% OsO_4 in phosphate buffer, dehydrated in an ethanol series, and embedded in Epon. Ultrathin sections were cut and stained with 7% uranyl acetate in methanol and lead citrate. They were examined under a 1 200 EX Jeol electron microscope, and the mineralized areas were analyzed with an energy-dispersive spectrometer (EDS Tracor). The accelerating voltage was 100 KeV and the current was chosen to limit the damage caused by the beam.

For calcium and phosphorus assays, minerals were solubilized with 5% perchloric acid. The calcium (Ca) concentration was measured by flame ionization (Spectrometer Eppendorf) and phosphorus was assayed by the molybdate vanadate reaction (Boehringer Mannheim, Germany, n°124 974).

Statistical Analysis

All experiments were done in triplicate. The results are expressed as mean ± standard deviation. Statistical analysis using the Mann-Whitney U test determined the difference in medians. Significance was assigned at the $P < 0.05$ level.

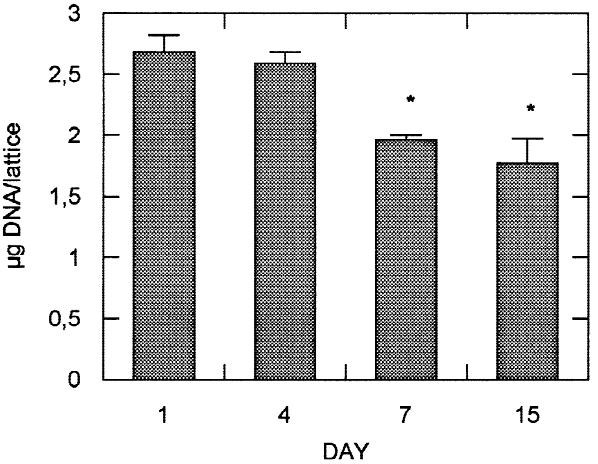

Fig. 1. Proliferation of human osteoblasts in collagen type I gel. Lattices were prepared as described under Methods in medium without βGP. On the days indicated, cells were harvested for determination of total DNA by fluorimetric assay with bisbenzimide (*$P < 0.05$ compared with day 4).

Results

Cell Growth and Viability

The proliferative state of osteoblasts grown in gels without βGP was studied by DNA measurement. The results (Fig. 1) showed no increase in the DNA content per lattice during the culture, with a slight decrease in DNA observed from day 7. Cell cycle was monitored along the cultures. After 2 days, 13% of the cells were in the S phase. This ratio decreased to 8% at day 5. At day 13, 5.5% of the cells were in the S phase, and most of the cells were arrested in the G0G1 phase (91.5%). This profile was close to that of subconfluent monolayers on plastic substrate (data not shown).

Cell viability was checked by quantification of CFDA-positive cells. Figure 2 shows one representative graph ob-

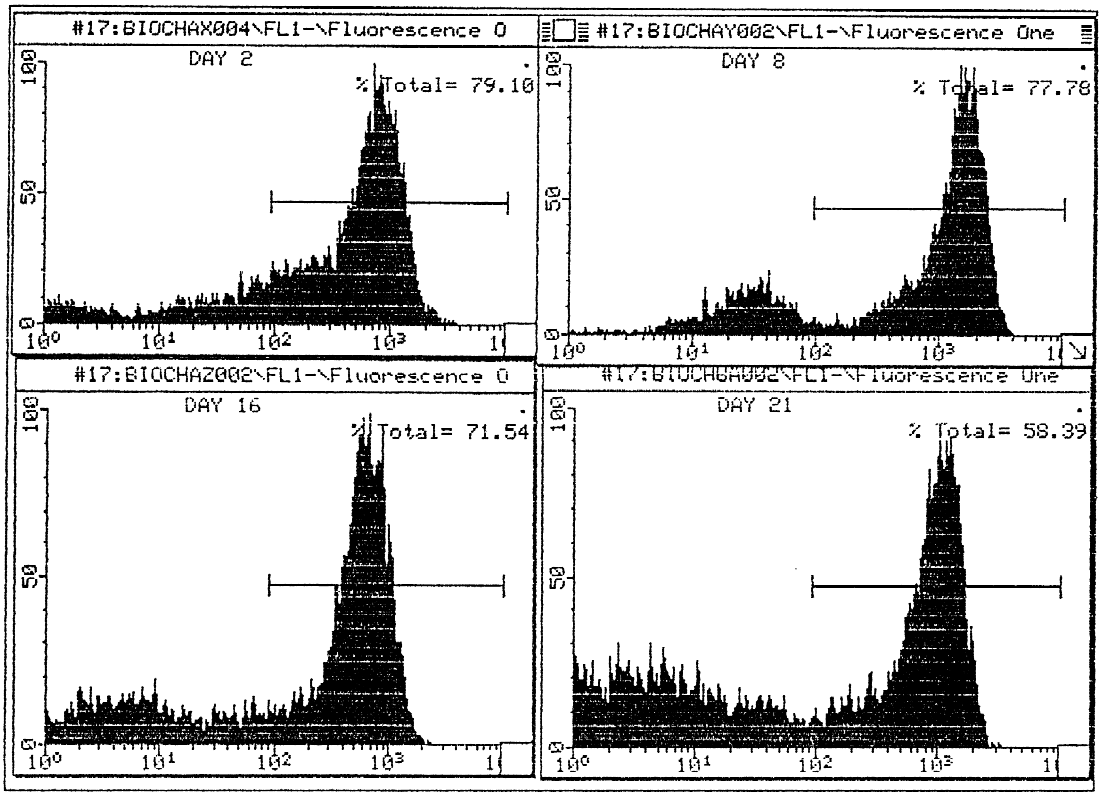

Fig. 2. Viability of osteoblast cells grown in a collagen gel for 2 days, 8 days, 16 days and 21 days. Viability was expressed as the percent of cells stained by CFDA. Fluorescence was measured by FACS analysis. This histogram was representative of three experiments.

tained by cytoflux analysis. The mean for three experiments showed 79.9% ± 1.7 of viable cells at day 2, 78.0% ± 2.29 at day 8 (statistically no different from day 2), and a significant decrease of viability seen from day 16: 70.1% ± 1.49 ($P < 0.05$) at day 16 and 56.5% ± 1.88 ($P < 0.05$) at day 21.

Mineralization

Human osteoblastic cells were seeded in a collagen gel and fed with 10 mM βGP from the beginning. Von Kossa staining performed at days 15 and 30 showed intense and homogenous mineralization within the lattice sections (Fig. 3). Staining was darker at day 30 versus day 15 (Fig. 3B). Von Kossa staining was negative when βGP was absent (Fig. 3A). The mineral deposits were analyzed by X-ray microanalysis. The spindle-shape crystallites observed on ultrathin sections with transmission electron microscopy were composed of Ca and P with atomic Ca/P ratios of 1.75 ± 0.38; the theoretical value for hydroxyapatite crystals is 1.67 (Fig. 4).

The involvement of the osteoblasts in mineralization was checked by comparing lattices populated with osteoblasts with lattices populated with human dermal fibroblasts. Ca and P were measured by chemical assays (Table 1). No mineralization occurred in the fibroblasts system up to day 16, and the calcium content of the fibroblast lattices was less than 10% of that in osteoblast lattices at day 23. The Ca/P ratio was low (1.09), and significantly lower than Ca/P ratio in osteoblast-populated lattices.

Effect of βGP on Gel Contraction

The effects of βGP on gel contraction were examined with βGP added to the initial lattice mixture. The rate of the collagen gel contraction by osteoblasts was affected by the presence of βGP (Fig. 5). Contraction was rapid during the first 20 hours, and the diameter of the gel stabilized at one-third of the initial diameter when βGP was added; gel diameter continued to decrease, reaching almost one-fifth of the initial size in the controls without βGP.

Effects of Various βGP Treatemts on Mineralization

As mineralization requires βGP, we examined the influence of 10 mM βGP added either during lattice preparation or at various times during cell culture.

In the experiment presented in Table 2 we compared giving βGP from the beginning of the culture to giving it only on days 8 or 21; mineralization was measured after 29 days of culture. No mineralization occurred in the absence of βGP. βGP addition at day 21 did not trigger mineralization, whereas adding it at day 8 promoted slight mineralization (0.53 μmol Ca per lattice). When βGP was present throughout the culture, the amount of Ca per lattice was at the highest level (10.3 μmol per lattice).

When βGP was added from the beginning of the culture, mineral formation was measured throughout the culture period (Fig. 6A). Minerals were detectable from day 8 and increased until day 23 in lattices containing 200,000 cells. Minerals were measurable as early as day 3 in lattices pre-

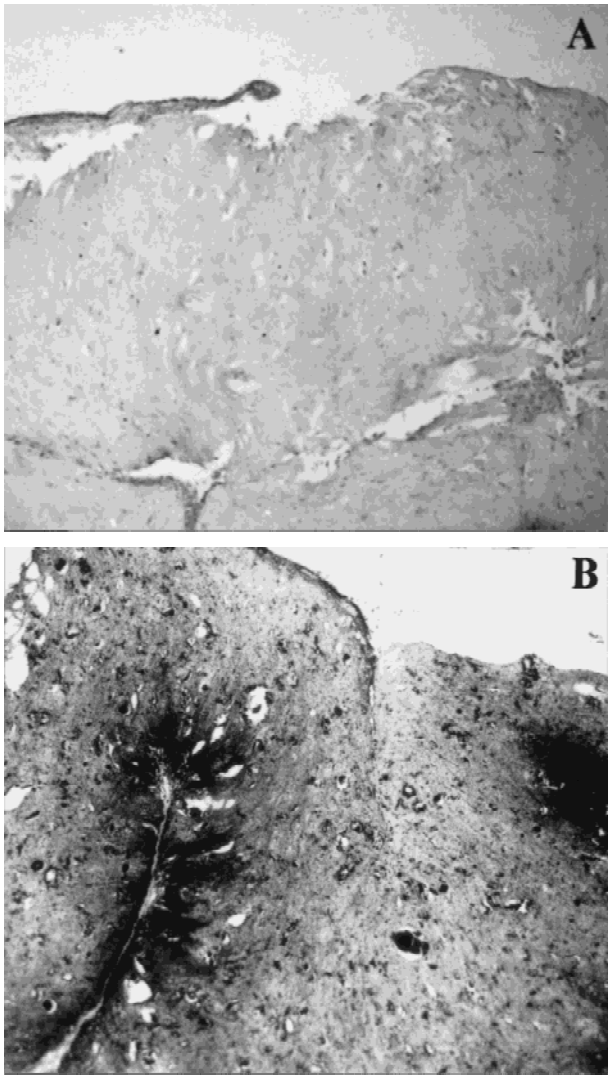

Fig. 3. Light microscopy of Von Kossa-stained sections from human osteoblast cells grown in collagen lattice for 15 days. **(A)** Without βGP, no dark deposits were seen; **(B)** with 10 mM βGP, large areas were stained indicating mineral repartition. Original magnification ×40.

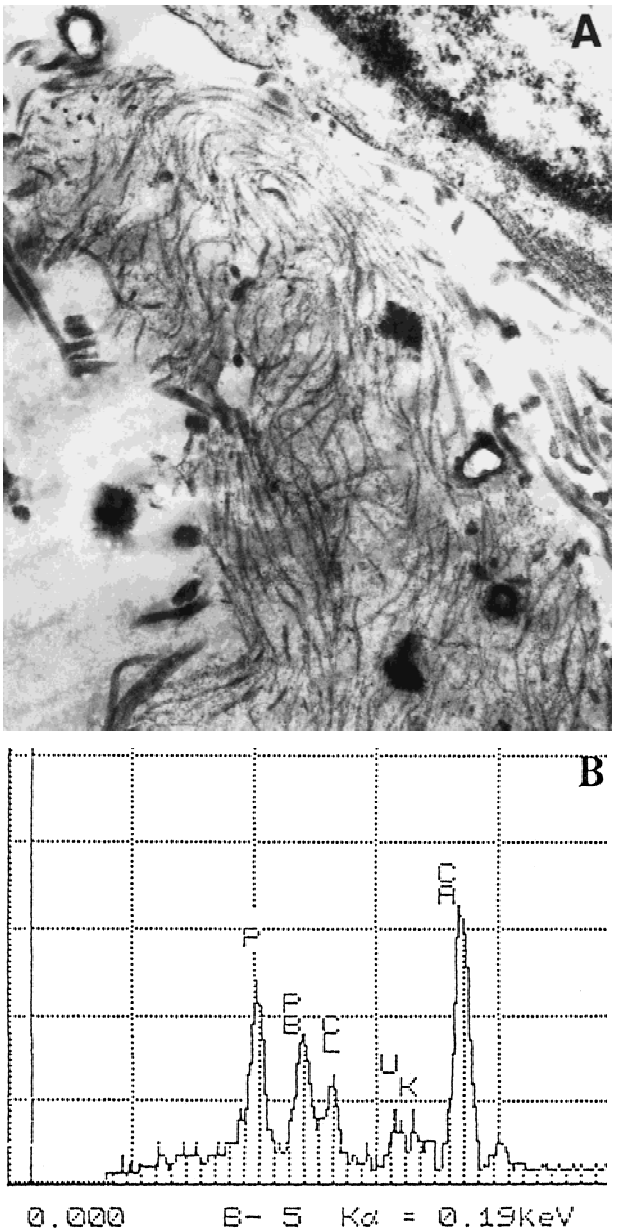

Fig. 4. X-ray microanalysis of mineral. **(A)** Electron-dense needle-like crystals in lattices after 3 weeks culture of osteoblast cells; original magnification ×30,000. Note the presence of large urchin-like structures within collagen network as well as some electron-dense collagen fibers on the left of the panel. **(B)** Spectrogram of crystals with calcium and phosphorus obtained with EDS.

pared with three times more collagen and containing 600,000 osteoblasts (0.163 ± 0.027 μmol Ca per lattice). The most favorable conditions for induction of mineralization were also tested by giving βGP as a single administration from day 0 to day 3. This was sufficient to induce mineralization with an increase in Ca deposition until 15 days of culture (Fig. 6B).

Effect of βGP on ALP Activity: Comparison of 2-D and 3-D

ALP activity in lattice cultures was assessed in comparison with cells grown in monolayers in the presence of βGP (Table 3). βGP had no effect on ALP activity in monolayer cultures. In contrast, it induced a large decrease in ALP activity in lattice cultures. In these lattices, ALP activity was only 50% of that of cultures grown without βGP as early as the second day of culture. It is worthy to note that ALP activity decreased during culture even in the absence of βGP.

Influence of Blocking Lattice Retraction

Free retracting gels were compared with nonretracting gels (Table 4). In both systems, mineralization, consequent to addition of βGP from day 0, was initiated between days 3 and 9. Accumulation of minerals seemed to progress inde-

Table 1. Mineral deposits in collagen lattices populated by human osteoblasts or fibroblasts in the presence of 10 mM βGP

Day	Cells	Ca μmol/lattice	Phosphorus μmol/lattice	Ca/P
2	Fibroblasts	0	0	/
	Osteoblasts	0	0	/
8	Fibroblasts	0	0	/
	Osteoblasts	2.66 ± 0.39	1.46 ± 0.17	1.82 ± 0.05
16	Fibroblasts	0	0.06	/
	Osteoblasts	5.46 ± 0.56	3.37 ± 0.13	1.61 ± 0.10
23	Fibroblasts	1.16 ± 0.35	1.06 ± 0.20	1.09 ± 0.12
	Osteoblasts	10.93 ± 1.5[a]	6.54 ± 0.76[a]	1.67 ± 0.04[a]

Values are means ± SD of three samples each
[a] $P < 0.05$ compared with fibroblasts on the same day

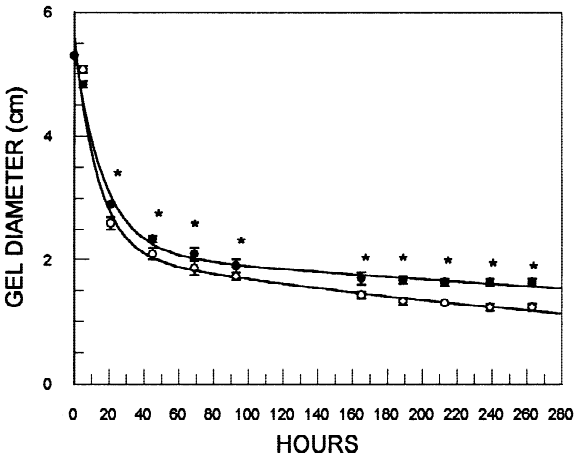

Fig. 5. Contraction rate of collagen gel populated with human osteoblasts and grown in MEM-10% newborn calf serum, □ without βGP, ■ with 10 mM βGP. Data were representative of three separate experiments and were expressed as the mean ± SD of triplicate gels (*$P < 0.05$).

pendently of ALP activity. Ca/P contents were comparable in free gels and in fixed gels, at days 9 and 15. In both systems, ALP activity decreased with time, but this decrease was slower in nonretracting gels: in free-floating gels, ALP activity at day 9 had decreased by 97% whereas it had decreased by 90% in nonretracting gels as compared with day 3.

Discussion

Concerning the behavior of the osteoblasts in the three-dimensional type I collagen gel, cells rapidly stopped dividing and the majority of cells were arrested in the G0G1 phase. The proliferative behavior of osteoblasts in free-floating type I collagen gels was similar to that of fibroblasts, with very little proliferation [23, 24] and an accumulation of fibroblasts in the G0G1 phase due to their interaction with collagen [25]. Talley-Ronsholdt et al. [26], who have cultured osteoblasts in a 3-D system, found that the osteoblasts exhibited growth, although the rate of multiplication was lower than in monolayers. However, their experimental model differed from ours: they used cells from neonatal rat calvaria, and cell suspensions obtained by enzymatic dissociation were placed directly in the gel; in our system, we subcultured to eliminate contaminant cells. Others studies were carried out with human osteoblastic cells from normal or osteosarcoma origin seeded onto a collagen gel [27, 28]. These systems allowed a cellular growth but with a reduced speed compared with plastic support. It is likely that some of the cells were migrating inside the gels, while cells remaining onto the surface continued to proliferate. In this work, we reported that adult human normal osteoblasts seeded into a collagen gel were not proliferative. These results support using this system because cells were quiescent.

The decrease in cell viability observed from day 15 could be attributed to the small size of the retracted gels in which cells were confined. Osteoblasts and fibroblasts were able to retract the gels very rapidly and very completely. For this reason we chose human dermal fibroblasts as negative controls to check the specificity of mineralization. Indeed, it was previously described that high phosphate concentrations in the culture medium can precipitate Ca in the absence of osteoblasts under appropriate conditions [13, 29]. Therefore, fibroblastic controls were used rather than gels without cells because fibroblasts also contract the gel at about the same rate and form a tissue-like lattice similar to that formed by osteoblasts, whereas gels without cells were very loose and cannot be compared with cell-populated gels. Phosphorus and calcium were only detectable in fibroblast controls after 23 days in culture, and accounted for less than one-seventh of the osteoblast production under the same conditions. The Ca/P ratio of these controls was also lower than the ratio in osteoblasts, and was not characteristic of hydroxyapatite (Table 1). These results are explained by a low endogenous ALP activity in fibroblasts which did not allow the release of inorganic phosphorus from βGP. Lastly, with electron microscopy and energy-dispersive spectrometry analysis no crystal deposits in fibroblast-populated lattices were found.

This work demonstrated that osteoblasts from human adults, seeded within a collagen type I gel, induced a mineralization process in the presence of βGP. Some urchin-shaped mineral particles deposited in the collagen network were identified as hydroxyapatite crystals by electron probe analysis. Mineral deposition was also observed along collagen fibers. We have previously shown that these cells could mineralize the newly synthesized extracellular matrix after long-term (at least 20 days) cultures on plastic substrate [16]. We showed here that the mineralization process occurred during the first week. Mineralization was quantifiable from day 8 in a gel containing 200,000 cells and from day 3 in gels with 600,000 cells.

In all the published *in vitro* mineralization studies it was found that organic phosphates must be added to the osteoblast cultures as a source of inorganic phosphorus. βGP is usually used at 10 mM, corresponding to the plasmatic concentration. βGP is generally added on days 15–21 in monolayer cultures time, at which the extracellular matrix is formed in the presence of ascorbate, and nodules have appeared [10, 30]. βGP induced mineralization of the osteoid-like matrix only in the nodules [31]. The main interest of our system is that the lattice provides the 3-D environment prerequired for mineralization. Cultures grown on plastic for a long period develop multilayer nodules which provide 3-D organization needed for mineralization, including cell-matrix and cell-cell interaction. In contrast, in our system,

Table 2. Mineralization in collagen lattice supplemented with 10 mM βGP at different culture times

	Without βGP	βGP from day 21	βGP from day 8	βGP from day 0
μmol Calcium/ lattice at day 29	0.10 ± 0.07	0.08 ± 0.035	0.53 ± 0.098[a]	10.32 ± 0.848[a]
μmol Phosphorus/ lattice at day 29	0	0	0.279 ± 0.003[a]	6.12 ± 0.602[a]

Osteoblasts were seeded in collagen gel in the presence of 10 mM βGP either from the beginning of the culture or from day 8 or 21, then βGP was supplemented at each medium change. At day 29, lattices were proceeded for calcium and phosphorus assay as described in Materials and Methods. Data presented were mean ± SD of triplicate cultures
[a] $P < 0.05$ as compared with the control without βGP

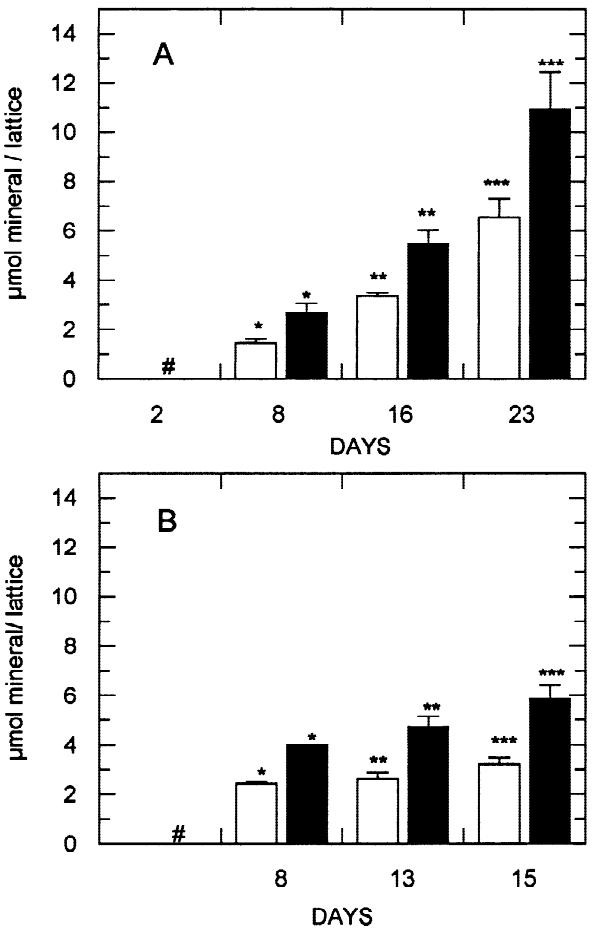

Fig. 6. Mineralization in 3-D osteoblast cell cultures. Open bar: phosphorus deposit, dark bar: calcium deposit. **(A)** βGP (10 mM) was added at the time of preparation of the lattice; medium plus βGP was replaced twice a week. **(B)** βGP (10 mM) was added at the time of preparation of the lattice, then culture medium was changed 3 days later, without βGP supplementation. #Ca and P undetectable; *$P < 0.05$ versus day 2; **$P < 0.05$ versus day 8; ***$P < 0.05$ versus day 16.

cell-cell contact was not much needed for inducing mineralization.

This raised the question of whether this exogenous matrix must mature before the βGP can induce mineralization. So we chose various times for the βGP supply: at the beginning of the culture, at the end of gel retraction (day 8), or later. βGP induced mineralization when it was added at the beginning of the culture, when cells expressed a high ALP activity. When βGP was added from day 8, a slight mineralization was seen, whereas nothing occurred later if βGP was added. As a consequence, a single administration of βGP at the beginning of the culture was sufficient to induce mineralization which increased thereafter. Furthermore, we showed that progression of mineralization did not require βGP nor ALP activity.

In monolayer cultures, the sequence of events leading to mineralization were well described [30, 31] and each sequence was conditional for the following stage. An extracellular matrix is prerequired for mineral deposition and especially type I collagen, which is the major extracellular matrix protein of bone. Our results in 2-D cultures were in agreement with those of Bellows et al. [31] with mineralization occurring after 17 days in culture. In contrast, our cultures in native type I collagen gel provided a suitable environment allowing an immediate mineralization process. It could be used to investigate the influence of effectors acting at the beginning of the culture, when mineralization is still controlled by osteoblasts.

Several studies have shown that bone cells can respond to strain *in vitro* and that the response is modulated by the way of exertion of the strain [32, 33]. In this work, we used a nonretracting gel model. In this case, continuous mechanical stress was generated by contraction of the osteoblasts themselves against the gel. The ALP activity seemed to decrease slightly slower in nonretracting gels, but no noticeable difference of mineralization was observed. In this system, two factors can be responsible for the modification of ALP activity: the effect of mechanical strain by itself and the 3-D environment. It is conceivable that modulation of ALP activity by collagen type I is secondary to the specific integrinic receptors interacting with the collagen network (personal data, unpublished), these integrins being differently expressed in free retracting gels and in nonretracting gels. In fact, osteoblasts were in a different 3-D environment in relation to the available space: in free-floating gels, the retraction was complete at day 9 and at this point the cells were in greater proximity. Cells in fixed gels were maintained isolated all through the experiment. The strain generated in this model was relatively small and further experiments are needed to determine how mechanical static strain on the collagen gels could modulate the phenotypic expression of human osteoblasts.

In conclusion, cultures of human osteoblastic cells grown in a network of native collagen gel rapidly form

Table 3. Modulation of ALP activity in human osteoblastic cell cultures by βGP medium supplementation

Culture model	Culture time days	ALP	
		Without βGP	With βGP
		mU/mg protein	
Monolayers			
Experiment A (n = 3)	15	991 ± 26.8	986 ± 21.6
Exp B (n = 3)	21	1005 ± 85.3	805 ± 25.4
Exp C (n = 4)	21	735 ± 63.6	709 ± 22.2
Exp D (n = 3)	21	885 ± 93.0	831 ± 54.7
		mU/lattice	
Lattices in	2	376 ± 15.5	141 ± 8.6[a]
MEM-10% FCS	8	61 ± 3.3[b]	7.7 ± 1.8[a,b]
(n = 3)	16	7.1 ± 1.8[c]	3.4 ± 0.0[a,c]

βGP (10 mM) was added from the beginning, and at each change. Each monolayer experiment was done with cells from donors of different sex and age
[a] $P < 0.05$ compared with lattices without βGP at the same day
[b] $P < 0.05$ compared with day 2 in the same culture medium
[c] $P < 0.05$ compared with day 8 in the same culture medium

Table 4. Effect of blocking retraction on mineralization by osteoblasts grown in collagen lattices

	Day	ALP mU/mg protein	Phosphorus μmol/lattice	Calcium μmol/lattice	Ca/P
Free floating	3	5573 ± 36.7	0	0	0
lattice in	9	196 ± 65.1[b]	2.97 ± 0.21	5.24 ± 0.21	1.76 ± 0.05
MEM-10% CS	15	165 ± 15.8	4.72 ± 0.19	8.34 ± 0.30	1.77 ± 0.02
Nonretracting	3	6956 ± 277.5[a]	0	0	0
lattice in	9	660 ± 152.1[a,b]	3.40 ± 0.33	6.14 ± 0.57	1.80 ± 0.03
MEM-10% CS	15	239 ± 40.3[c]	4.42 ± 0.25	7.98 ± 0.58	1.80 ± 0.03

Values are means ± SD of three samples. ALP activity was assayed on cell supernatant after ultrasonic lysis of the cells
[a] $P < 0.05$ compared with free-floating lattices on the same day
[b] $P < 0.05$ compared with day 3 in the same system
[c] $P < 0.05$ compared with day 9 in the same system

mineral deposits, via alkaline phosphatase. ALP induced the early stage of the mineralization process that further progressed by itself. These cultures are sensitive to their environment, and therefore responsive to the actions of effectors on the regulation of differentiation.

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References

1. Ecarot-Chavrier B, Glorieux FH, Van Der Rest M, Perreira G (1983) Osteoblasts isolated from mouse calvaria initiate matrix mineralization in culture. J Cell Biol 96:639–643
2. Hall BK, Dickson IR (1985) The effects of parathyroid hormone on osteoblast-like cells from embryonic chick calvaria. Acta Endocrinol 198:217–220
3. Schmid C, Steiner T, Froesch ER (1983) Insulin-like growth factors stimulate synthesis of nucleic acids and glycogen in cultured calvaria cells. Calcif Tissue Int 35:578–581
4. Gerstenfeld LC, Chipman SD, Glowacki J, Lian JB (1987) Expression of differentiated function by mineralizing cultures of chicken osteoblasts. Dev Biol 122:49–50
5. Clover J, Gowen M (1994) Are MG-63 and HOS TE85 human osteosarcoma cell lines representative models of the osteoblastic phenotype? Bone 15:585–591
6. Majeska RJ, Rodan GA (1982) The effects of 1,25(OH_2)D3 on alkaline phosphatase in osteosarcoma cells. J Biol Chem 257: 3362–3365
7. Rodan SB, Imai Y, Thiede MA, Wesolowski G, Thompson D, Bar-Smavit Z, Shull S, Mann K, Rodan EA (1987) Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer Res 47:4961–4966
8. Koshihara Y, Honda Y (1994) Age-related increase in collagen production in cultured human osteoblast-like periosteal cells. Mech Aging Dev 74:89–101
9. Robey PG, Termine JD (1985) Human bone cells in vitro. Calcif Tissue Int 37:453–460
10. Bellows CG, Aubin JE, Heersche JNM, Antosz ME (1986) Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int 38: 143–154
11. Tenenbaum HC (1981) Role of organic phosphate in mineralization of bone in vitro. J Dent Res 60:1586–1589
12. Bellows CG, Aubin JE, Heersche JNM (1991) Initiation and

progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. Bone Miner 14:27–40
13. Khouja HI, Bevington A, Kemp GJ, Russell RGG (1990) Calcium and orthophosphate deposits in vitro do not imply osteoblast-mediated mineralization: mineralization by betaglycerophosphate in the absence of osteoblasts. Bone 11:385–391
14. Tenenbaum HC (1987) Levamisole and inorganic pyrophosphate inhibit β-glycerophosphate-induced mineralization of bone formed in vitro. Bone Miner 3:13–26
15. Chung CH, Golub EE, Forbes E, Tokuoka T, Shapiro IM (1992) Mechanism of action of β-glycerophosphate on bone cell mineralization. Calcif Tissue Int 51:305–311
16. Rattner A, Sabido O, Massoubre C, Rascle F, Frey J (1997) Characterization of human osteoblastic cells: influence of the culture conditions. In Vitro Cell Dev Biol Animal 33:757–762
17. Bell E, Ivarsson B, Merrill C (1979) Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci USA 76:1274–1278
18. Riikonen T, Koivisto L, Vihinen P, Heino J (1995) Transforming growth factor-β regulates collagen gel contraction by increasing α2β1 integrin expression in osteogenic cells. J Biol Chem 1:376–382
19. Frey J, Chamson A, Raby N, Rattner A (1995) Collagen bioassay by the contraction of fibroblast-populated collagen lattices. Biomaterials 16:139–143
20. Chapuis JF, Lucarz-Bietry A, Agache P, Humbert P (1996) A mechanical study of tense collagen lattices. Eur J Dermatol 6:56–60
21. Labarca C, Paigen K (1980) A simple rapid and sensitive DNA assay procedure. Anal Biochem 102:344–352
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
23. Nakagawa S, Pawelek P, Grinell F (1989) Long-term culture of fibroblasts in contracted collagen gels: effects on cell growth and biosynthetic activity. J Invest Dermatol 93:792–798
24. Nishiyama T, Tsunenaga M, Nakayama Y, Adachi E, Hayashi T (1989) Growth rate of human fibroblasts is repressed by the culture within reconstituted collagen matrix but not by the culture on the matrix. Matrix 9:193–199
25. Kono T, Tanii T, Furukawa M, Mizuno N, Kitajima J, Ishii M, Hamada T, Yoshizato K (1990) Cell cycle analysis of human dermal fibroblasts cultured on or in hydrated type I collagen lattices. Arch Dermatol Res 282:258–262
26. Talley-Ronsholdt EJ, Lajiness E, Nagodawithana K (1995) Transforming growth factor-beta inhibition of mineralization by neonatal rat osteoblasts in monolayer and collagen gel culture. In Vitro Cell Dev Biol Animal 31:274–282
27. Casser-Bette M, Murray AB, Closs EI, Erfle V, Schmidt J (1990) Bone formation by osteoblast-like cells in a three-dimensional cell culture. Calcif Tissue Int 46:46–56
28. Heermeier K, Spanner M, Träger J, Gradinger R, Schmidt J (1995) Bone formation of human osteoblast-like cells in a three-dimensional cell culture: an in vitro model for studies of skeletal disorders. Cells Mater 5:309–321
29. Marsh ME, Munne AM, Vogel JJ, Cui Y, Franceschi RT (1995) Mineralization of bone-like extracellular matrix in the absence of functional osteoblast. J Bone Miner Res 10:1635–1643
30. Sudo H, Komada HA, Amamgai Y, Yamamoto S, Kasai S (1983) In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol 96:191–198
31. Bellows GG, Heersche JNM, Aubin JE (1992) Inorganic phosphate added exogenously or released from β-glycerophosphate initiates mineralization of osteoid nodules in vitro. Bone Miner 17:15–29
32. Zhuang H, Wang W, Tahernia A, Levitz CL, Luchetti WT, Brighton CT (1996) Mechanical strain-induced proliferation of osteoblastic cells parallels increased TGF-β1 mRNA. Biochem Biophys Res Commun 229:449–453
33. Brighton CT, Strafford B, Gross SB, Leatherwood DF, Williams JL, Pollack SR (1991) The proliferative and synthetic response of isolated calvarial bone cells of rats by cyclic biaxial mechanical strain. J Bone Joint Surg 73:320–331