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Selection of Malonate-Resistant Stromal Cell-Derived Osteoprogenitor Cells In Vitro

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Abstract Bone marrow stromal cells give rise to osteoprogenitor cell (OPC) colonies, with characteristic mineralized bone nodules in vitro. During differentiation, OPCs in the culture are surrounded by heterogeneous populations of various cell lineages and by different OPC differentiation stages In the present study, attempts were made to increase the homogeneity of OPCs in culture The reliance on energy metabolism restricted to glycolysis, which is specific to the premineralizing skeletal cells, was tested as a selectable marker for cells in this stage Day 12 alkaline phosphatase (ALP) and day 20–21 calcium precipitates were used as early and late OPC differentiation markers Malonate, a competitive inhibitor of succinate dehydrogenase, was added to the OPC stimulation medium, to interfere with the Krebs cycle-dependent energy metabolism operating in most of the stromal cells OPCs that entered the stage of energy metabolism restricted to glycolysis were expected to become malonate resistant Malonate showed dose and time dependence, 10 mM malonate added on day 3, decreased day 12 ALP activity/well to the lowest level Variations in time and length of exposure to malonate used during the first 12 days of differentiation showed an inverse correlation between specific ALP activity and cell yield. Malonate-treated variations of specific ALP and of cell yield indices were up to 30- to 40-fold larger than variations within day 21 calcium precipitates. Thus, calcifying cells were almost unchanged relatively to noncalcifying cells. These results indicate that malonate-resistant cells are mostly selected, rather than induced, to differentiate by malonate. The results also show that stromal derived OPCs undergo a similar biochemical stage as in chondrocytes

Key words: energy metabolism, glycolysis, differentiation stage, alkaline phosphatase, mineralization

During differentiation, skeletal cells undergo a transient biochemical alteration, their microenvironment becomes hypoxic [Brighton and Heppenstall, 1971], and their redox state is changing [Shapiro et al., 1982]. During hypoxia observed at the growth plate microenvironment, the chondrocytes rely on glycolysis as a source of energy. This biochemical alteration is characteristic of a differentiation stage in these cells and should provide an opportunity to enrich selectively osteoprogenitor cells (OPCs) at their "anaerobic" stage relative to the surrounding majority of non-OPCs that use more energy from "aerobic" metabolism. Hypothetically, at their anaerobic glycolytic stage, unlike other cells, OPCs should be selectively resistant to blockers of the "postglycolytic" pathway.

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The aerobic energy metabolism depends on reactions catalyzed by mitochondrial enzymes of the citric acid cycle. Therefore diminishing the citric acid cycle efficiency by blocking key enzymes is expected to arrest cell proliferation and cellular functions of non-OPCs or OPCs in their aerobic stage.

The mitochondria, in which the citrate acid cycle occurs, also participates in controling the cellular calcium metabolism in both soft and hard tissues. The mitochondria in various soft tissues accumulates calcium [Carafoli and Sottocasa, 1984] by a metabolism-dependent process, but mitochondria in skeletal cells presumably have an additional mechanism for calcium binding [Shapiro and Lee, 1975]. It was also found that calcium precipitation in the growth plate is preceded by calcium efflux from mitochondria [Brighton and Hunt, 1978]. Chondrocytic mitochondria accumulates calcium in a crystallized form [Lehninger, 1970], which is released when cartilage mineralization takes place. The mechanism whereby calcium is ex-

truded from skeletal cell mitochondria has not been completely determined yet, but studies in the past have elucidated part of this process. It is known for long that cell under low oxygen tension restrict their energy utilization to the premitochondrial glucolytic pathway (anaerobic metabolism) [Krebs, 1953]. A shift to glycolytic metabolism has indeed been observed between the proliferative zone and the hypertrophic condrocytes before entering to the mineralizing front in the growth plate [Brighton et al., 1969]. Under anaerobic conditions, molecular oxygen is less available in the mitochondria to cooperate with oxidative phosphorylation and extrusion of NADH-derived protons. Accordingly, it has been found that under anaerobic conditions, in the hypertrophic chondrocytes of the growth plate, the NADH/NAD ratio is elevated [Shapiro, 1982]. Earlier it was found that a lower cytosolic pH and accumulation of phosphoenol pyruvate, both characteristic of a glycolytic anaerobic metabolism, are associated with calcium release from chondrocytic mytochondria [Shapiro and Lee, 1978]. Also the stroma derived OPCs presumably undergo a stage with characteristics of anaerobic metabolism during stromal cell differentiation. Stromal cells constitute a heterogeneous population [Owen et al., 1987]; furthermore, OPCs themselves at any time in culture constitute a heterogeneous population [Rodan et al., 1988]. In order to analyse a homogeneous population of a single OPC differentiation stage, it is necessary to enrich the cell population with this particular stage. In the present publication, we describe the effect of sodium malonate, a competitive inhibitor of succinate dehydrogenase, on the relative enrichment of stromal cell-derived OPCs in culture.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, sodium malonate, dexamethasone, ascorbate, β-glycerophosphate, Alizarin Red S, Light Green SF Yellowish were purchased from Sigma, St. Louis, MO. Fetal calf serum (FCS) was purchased from Grand Island Biological Company, N.Y.

Stromal Cell Culture

Bone marrow cell suspensions obtained from femurs and tibiae of female Sabra rats, weighing 60–80 g, were seeded in 25-cm^2 flasks, 10^8 cells/flask. The stroma cells were obtained as described by [Maniatopoulos et al., 1988], removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium, that consisted of DMEM (Dulbecco modified Eagle's medium) supplemented with 15% fetal calf serum and antibiotics, in a humid 10% CO_2 atmosphere. For the experimental cultures stroma cells were removed 2 weeks later by trypsinization and were plated in microtiter plates, 5,000 cells/well and grown in osteoprogenitor cell (OPC) stimulation medium. This consisted of maintenance medium containing 10^{-8} M dexamethasone, 50 μg ascorbate/ml and 10 mM β-glycerophosphate.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. ALP activity in primary bone cell cultures was shown to peak between days 10 and 12 [Deren et al., 1990]; therefore, day 12 of dexamethasone stimulation was set for ALP assay and cell count. Growth medium was removed, and the cells were washed twice in situ with 0.2 ml TNC (50 mM Tris, 150 mM NaCl pH 7.6). ALP substrate, pNPP (p-nitrophenyl phosphate) in TNC, 1.33 mg/ml, was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min, and the optical density of the hydrolysed pNPP was measured in a multichannel spectrophotometer at 405-nm wavelength. ALP activity was expressed either as nMol/time/cell (or per well) or as O.D. units. For experiments that required propagation of the same cells in culture, after undergoing an in situ ALP assay, the procedure was carried out under sterile conditions. A translucent sterile coverslip was placed on the plates during the spectrophotometry. Immediately following O.D. determination the substrate was replaced by growth medium, after the cells were washed once with TNC, to continue their propagation in culture.

For a study of the levamisole effect on stromal differentiation (submitted), it was necessary to determine whether in situ ALP activity at pH 7.6 correlates with the activity at pH 10.3. The assay at both pH conditions was performed in tandem in each plate. The linear regression for cells grown in OPC inductive medium showed a direct correlation ($r = 0.839$) between phosphatase assays at both pH conditions, this is acceptable considering the nature of such a cellular assay. The linear regression of the enzyme activities in cells cultivated in the presence of le-

vamisol, showed an even better correlation (r = 0.919). This result indicates that 90-min incubation at pH 7.6 is adequate for comparative ALP assays if groups with multiple samples are being compared.

Quantitative Cell Staining

After the ALP assay, in some of the experiments, cells were stained using the methylene blue (MB) method [Goldman and Bar-Shavit, 1979]. The cells were fixed in 0.5% glutaraldehyde for 30 min, rinsed with D H_2O, and air-dried overnight. Borate buffer (0.1 M boric acid brought to pH 8.5 with NaOH) 0.2 ml/well, was added to the cells for 2 min and rinsed with tap water. Cells were then incubated in 0.1 ml of 1% MB in borate buffer for 60 min at room temperature, rinsed with water, and air dried. The MB was then eluted from the stained cells by incubation with 0.2 ml of 0.1 N HCl at 37°C for 60 min. The O.D. of the eluted MB was measured at 620 nm by a multichannel spectrophotometer; 1.0 unit is equivalent to 5×10^4 stromal cells.

Measurement of In Vitro Precipitated Calcium

After 2 weeks of culture in OPC stimulation, medium calcium precipitates began to appear around some of the stroma cells, demonstrable by Alizarin Red as an orange-red staining. To quantitate these precipitates plates were washed twice with TNC and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations. The precipitates were expressed as μg Ca/well.

Alizarin Red S Staining

For a semiquantitative demonstration of mineralization, 20- to 21-day-old stimulated cultures were fixed 20 min in undiluted methanol. Subsequently 1.0 g Alizarin sodium sulfate (Sigma A-3757) in an aqueous solution of 0.1% NH_4OH was applied to the cultures for 2 min and then washed 3 times with distilled water. The cultures were counter stained for 2 min with 1% Light Green SF Yellowish (Sigma L-5382) in 1% acetic acid. Cultures were air-dried after rinsing with distilled water.

RESULTS

Determination of ALP Activity During Stromal Cell Differentiation

Before testing the ability of malonate to affect stromal cell commitment to day 21 mineralization (as related to ALP activity), determination of the appropriate day for ALP assay was required. Stromal cells were cultured with OPC stimulation medium containing dexamethasone, ascorbate, and βGP. ALP activity and cell

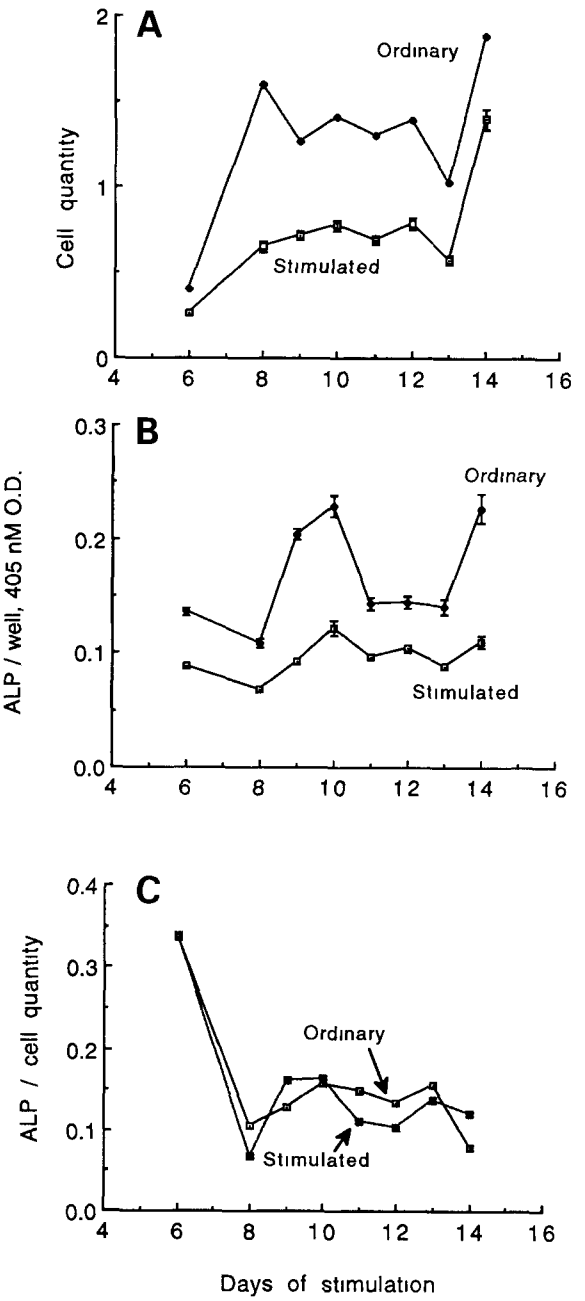

Fig. 1. Effect of OPC stimulation medium on stromal cell quantity, on ALP activity, and on specific ALP activity On day (−)3, microtiter wells were seeded with 3×10^3 cells/well and on day 0 the medium was changed to OPC stimulation medium ALP activity was measured and the methylene blue cell count was performed at several time intervals, a separate plate was assigned to each time point Each point represents the mean ±SE of 20 microtiter wells

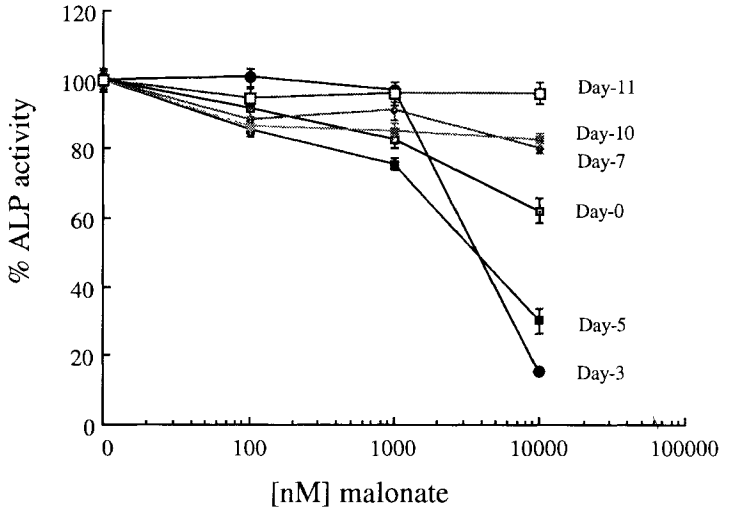

Fig. 2. Malonate effect on day 12 ALP activity in stimulated stromal cells. Stromal cells were grown in OPC stimulation medium and in different concentrations of malonate (from 10.0 mM to 100 nM). Malonate was added to the cultures at different days (as indicated near each curve) and removed on day 12. ALP activity was measured on day 12. Each point represents the mean ±SE of 24 wells.

quantitation were performed during differentiation at different time intervals. Figure 1A,B shows that the stimulation medium diminishes the absolute ALP activity and cell quantity, respectively. The specific ALP activity (Fig. 1C) expressed per cell on day 9 was only 25% higher, and on day 12 it was even lower than in the control cultures. The specific ALP activity in this system is not a net representative of all ALP-expressing osteogenic cells. This is because not all the osteogenic cells show an increased ALP activity at every instant during the differentiation period; also, some cells not associated with mineralization express ALP [Kamalia et al., 1992]. However, it is reasonable that ALP activity in a greater portion of the stimulated cultures is attributed to high ALP-expressing osteogenic cells, compared with the unstimulated cultures. Conversely, a diminishing portion of ALP activity is attributed to nonosteogenic cells. The OPC stimulation medium induces increased mineralization by increasing the number of osteogenic cell colonies. Calcium precipitation is detected in our system from day 14; thereafter (data not shown), this timing was also demonstrated in cultured rat calvarial osteoblasts [Pockwinse et al., 1992].

ALP Response to Malonate

Sodium malonate was added at different concentrations and time intervals, to several stromal cell cultures grown with OPC stimulation medium. The addition of malonate (Fig. 2) resulted in a low day 12 ALP activity; the dose with the highest effect on absolute ALP/well was 10 mM, being most prominent when added on day 3. Day 12 was chosen for ALP assays in this experiment based on its elevation in studies on osteogenic cells [Deren et al., 1991]. The diminished ALP activity was not due to inhibition of enzyme function, but rather to a diminished day 12 cell population observed by microscopy (Fig. 5A).

Correlation Between Day 21 Calcium Precipitate With Earlier ALP Activity

Days 20–21 were chosen for measuring cell-mediated calcium precipitates, because at that time they are detectable by Alizarin staining and by atomic absorption. Previous studies on stromal cell mineralization inhibition under the continuous presence of levamisole (submitted) suggested that inhibition of day 21 mineralization has at least two components, one consists of inhibition of enzymatic activity during crystal growth (a late stage controlled by ALP), the other component is an early inhibitory effect induced by levamisole during the first week in culture, before mineralization takes place. Therefore, the present study required determination of appropriate days during differentiation in which the ALP activity shows the best correlation with day 21 mineralization. For this pur-

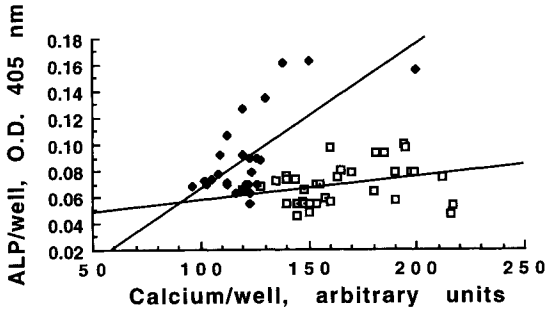

Fig. 3. Correlations between day 12 ALP activity and day 21 calcium precipitation of malonate-treated and untreated stroma Stromal cells were cultured from day 0 with OPC stimulation medium in the presence (empty squares) and absence (closed diamonds) of 10 mM malonate, which was added on day 3 and removed on day 12 ALP assay was performed on day 12 under sterile conditions at pH 7 6, subsequently, the cells of both groups were cultivated in OPC stimulation medium until day 21 The correlation coefficient of malonate treated and untreated cultures is r = 0 295 and 0 670, respectively

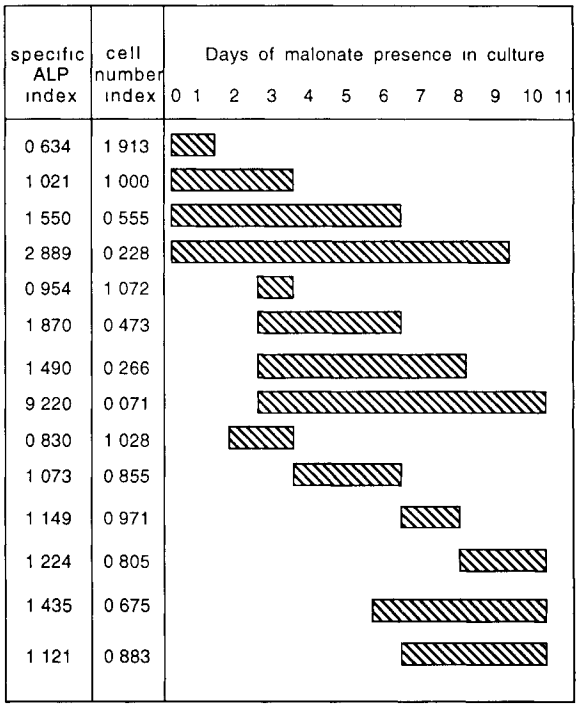

Fig. 4. Disection of the effect of malonate on stromal cells during the first 11 days of differentiation and proliferation Stromal cells were stimulated with OPC stimulation medium from day 0 and malonate was added on different days and removed on different days On day 11, specific ALP activity and cell quantity were determined and ratios of the values of cells cultivated with/without malonate are expressed as specific ALP index and cell No index For the mean of each ratio, n = 20

pose, stromal cells were stimulated in culture and individual wells of 3 plates underwent in situ ALP assay, each on a different day (days 6, 9, and 12). Malonate was added to one-half of the wells in each of these plates, for a final concentration of 10 mM on day 3, as inferred from the dose response presented in Figure 2. Malonate was removed before the ALP assay and was not returned thereafter. Immediately after the ALP assay, the cells continued to grow in OPC stimulation medium (without malonate) and on day 21, calcium precipitates were measured. Figure 3 illustrates the regression lines of malonate-treated and untreated cultures of stromal cells grown in OPC stimulation medium. Each point represents day 12 ALP plotted against day 21 calcium of the same individual well. One of the two linear regressions in Figure 3 (the line with the lesser slope) represents malonate-treated cells. In the cultures without malonate, the correlation of day 21 Ca with day 12 ALP activity ($r = 0.67$) is better than with day 6 and 9 ALP activity ($r = 0.044$ and 0.219, respectively). It is also slightly better than the correlation with ALP of day 21 (where r was close to 0.6). These results justify the use of day 12 ALP activity as a differentiation marker that relates to cell-mediated calcification measured on day 21. Day 12 ALP in this study fulfills a function of a differentiation marker, independent of the direct enzymatic role in calcification played by ALP on day 21.

The Effect of Malonate on the Differentiating Stromal Cell Population

To determine the time ranges during differentiation at which malonate has the highest positive effect on osteoprogenitor cell (OPC) yield and the highest negative effect on non-OPC yield, 10 mM malonate was added and removed from the cultures at different days. OPC differentiation was determined on day 12, by measuring ALP activity/cell (specific ALP). Figure 4 illustrates various time ranges at which the cultures were exposed to 10 mM malonate, as well as its effect on OPC differentiation and on stromal cell proliferation. Results are expressed as the ratio (index) between malonate-treated and untreated controls. The lowest OPC yield (specific ALP index = 0.634) was associated with the highest cell proliferation (cell No. index = 1.913) and was obtained when cultures were exposed to malonate only on days 0–1 (upper line in Fig. 4). The increase in OPC yield was directly related to the duration of the expo-

sure to malonate. When malonate was added on day 3 of OPC stimulation and maintained only for 1 day, a higher OPC yield was observed (specific ALP index = 0.954), and it was associated with a lower cell proliferation (cell No. index = 1.072). The highest OPC yield (specific ALP index = 9.22) was obtained when malonate was added on day 3 of stromal OPC stimulation and maintained up to day 11; this was associated with the lowest cell yield compared to other cultures (cell No. index = 0.071).

TABLE I. Comparison Between Effects of Malonate on Day 21 Calcium Precipitation With the Effects on the Differentiated OPC Yield (n = 20)

Day malonate added to culture	Day 0	Day 3	Day 5	Day 7
Day 21* calcium index	1 202	0.976	1 148	1 209
Day 12 cell no index	0 228	0.071	0 675	0 883
Day 12-specific ALP index	2 889	9 220	1 435	1 121

*Malonate removed on day 12

The Early Malonate Effect on Day 21 Cell-Mediated Mineralization

Malonate was added to differentiating stromal cells on day 0 from the start of OPC stimulation for a 12-day exposure and at three different time intervals, with diminishing length of exposure. Malonate was removed from the cultures on day 12; after washing the cells with TNS, they were cultivated in OPC stimulation medium until Day-21 for quantitation of acid-soluble calcium precipitates. Table I shows the calcium index/well aligned with indices of specific ALP and cell No./well of proper malonate exposure durations, taken from Figure 4. Calcium precipitates

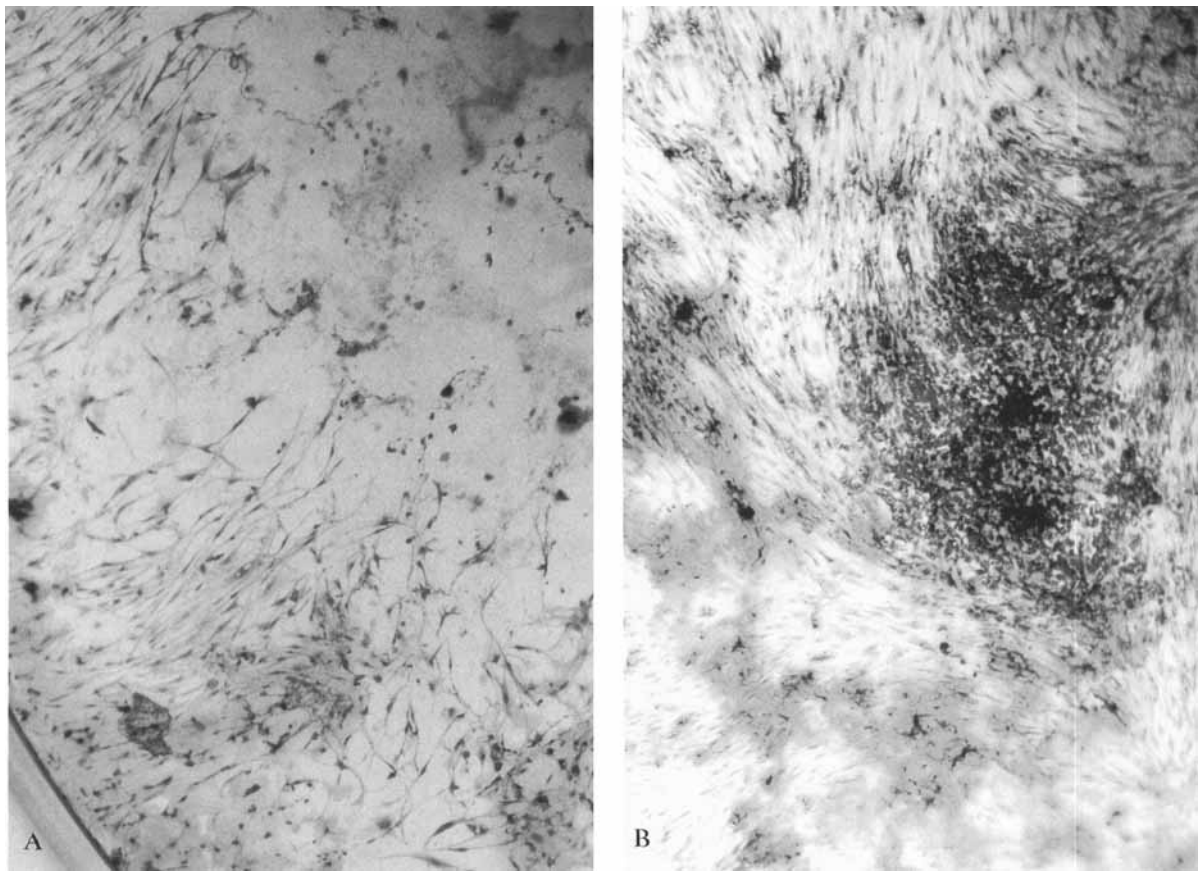

Fig. 5. Alizarin Red staining of day 21 calcium precipitates in stromal cell cultures cultivated in OPC stimulation medium **A:** Cells were grown in 10 mM malonate from day 3 (A) to day 9 (see Fig 4, line 7) **B:** Controls were grown without malonate Cells in the regions of relatively dense cellularity were counted, and the ratio of the mean counts from 5A and 5B is comparable to the cell No index on day 12 (see text) ×100

sustained minor changes in the presence of malonate relative to the major changes in the specific ALP/well and in cell quantities/well. This is consistent with the hypothesis that malonate selectively arrested the proliferation of non-OPCs but permitted the growth or survival of OPCs.

The most prominent (1400%) variable seen at the presence of malonate was the cell No. index observed when malonate was added on day 3, it was associated with a minimal effect (20–30%) on day 21 calcium index. This suggests that, in the cultures, on day 3, there is a peak of malonate-resistant OPCs that can tolerate malonate for up to 9 days, probably under continuous accumulation of such newly differentiated OPCs.

Microscopic View of Malonate Effect on Stromal Cell Quantity

Two opposing wells, one treated with malonate from day 3 for 6 days. The other untreated controls (Fig. 5A,B, respectively) were stained with Alizarin Red and with Light Green on day 21. In both wells there are regions with dens cell growth and regions with lower cell density. In the malonate-treated culture (Fig. 5A), the "crowded" regions contained 10.105 ± 3.11 cells/cm^2, whereas in the control (5b), they contained 47.555 ± 7.689 cells/cm^2. The ratio between these cell counts is 0.212, close to the cell No. index of 0.266 of the appropriate sample in Figure 4 (line 7).

DISCUSSION

In the present work, we examined the effect of malonate on the proliferation of stromal cells and on their differentiation in vitro. Based on studies with differentiating bone cells, ALP quantitation was performed on day 12. Dose-response analysis showed that 10 mM malonate is the appropriate concentration for lowering the absolute ALP activity/well on day 12. The diminished absolute ALP activity was later found to reflect a diminished stromal cell number and the cells even showed increased specific ALP activity/cell contrary to the decreased absolute activity/well.

In nonstimulated stromal cell cultures, specific ALP activity peaks were observed on days 6, 9, and 12, corresponding to days 3, 6, and 9 of cultures grown in dexamethasone-containing osteoprogenitor cell (OPC) stimulation medium. In dexamethasone-stimulated cultures, regression analysis between ALP activity/well (on days 6, 9, and 12) and day 21 precipitated-calcium revealed that the best correlation was obtained between day 12 ALP activity and day 21 calcium. These results indicated that, in differentiating stromal cells as well, day 12 is appropriate for the performance of the ALP assay and cell counting, if day 21 calcium results should be related to ALP. In stimulated stromal cells, calcium precipitates appear after 15 days in culture, but they become prominent on day 20 (data not shown). Therefore, calcium was always measured on days 20–21, although calcium may accumulate on bone nudules at least until day 35. This is in accord with results in calvarial osteoblasts [Pockwinse et al., 1992], which showed that 30% of maximal ALP expression and 30% of maximal mineralization occur on days 12 and 20, respectively.

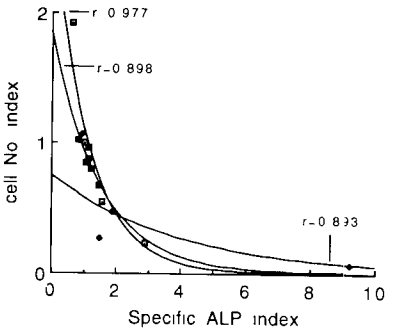

Fig. 6. Inverse correlation between relative values of malonate-selected stromal cell count and the relative specific ALP activity in selected cells Exponential regression lines of three separate groups of results taken from Figure 4 Open squares represent malonate added on day 0 and removed on different days, r = 0 977 (4 upper lines in Fig 4) Closed diamonds represent malonate added on day 3 and removed on different days thereafter, r = 0 893 (second four lines in Fig 4) Closed squares represent mostly samples of 2–3 days of exposure, r = 0 898 (last six lines in Fig 4) Each point represents the correlation between the mean of 20 index values Each index value represents the ratio between specific ALP (or cell count) in one well of malonate treated and its proper control well on the same plate

The various periods and the points of entry at which malonate was administered to the cultures during the first half of stromal cell differentiation revealed an inverted relation between specific ALP activity and total stromal cell count, both performed on day 12. Exponential regression lines (Fig. 6) obtained from three different groups of results, shown in Figure 4, show a sufficiently high correlation between these two parameters. The inverted correlation between differentiation (specific ALP) and proliferation (cell No.) in stromal cells is similar to the find-

TABLE II. Lack of Day 3 Malonate Ability to Substitute for Dexamethasone (DX) and Ascorbate (VC), as Mineralization Inducer in Stromal Cells

Culture no.	Ingredients in the OPC stimulation medium							Day 21 alizarin red staining
	Days 0–3		Days 3–12			Days 12–21		
	DX + VC	βGP[a]	DX + VC	βGP	Malonate	DX + VC	βGP	
1	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(4+)
2	(+)	(+)	(−)	(+)	(+)	(−)	(+)	Trace
3	(−)	(+)	(−)	(+)	(+)	(−)	(+)	(3+)
4	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)

[a]β-Glycerophosphate.

ings shown in differentiating osteoblasts [Stein et al., 1990]. Figure 6 shows that malonate treatment complys with and does not abrogate this inverted ratio.

The relative enrichment in OPCs caused by a longer presence of malonate is consistent with a selective effect of malonate favoring the OPC population over non-OPCs. This is supported by the fairly constant mineral quantities associated with different malonet entries and length of exposure. Based on the minimal changes in calcium, it is likely that malonate has mainly arrested the growth of non-OPCs with a weaker effect on the OPC population.

The experiments represented in Figures 2 and 4 suggest that the day of malonate addition also influences the relative enrichment of the cultures with ALP-expressing OPCs. Day 3 differs from other days by the stromal cell response to malonate. Malonate is most efficient on day 3 in enriching the cultures with OPCs of high ALP specific activity, as measured on day 12. Unexpectedly, adding malonate on day 3 is even better than adding it on day 0. The mechanism for this phenomenon is not clear; however, it can be used to test the conclusion that malonate has a selection rather than an induction effect on OPCs. Confirmation that the role played by malonate is restricted mainly to OPC selection was achieved by showing that ingredients in the stimulation medium are responsible for the induction of this phenomenon and that they can not be substituted by malonate. Table II shows that when dexamethasone and ascorbate are used for OPC stimulation starting on day 0, they must be maintained beyond day 3, since their removal on day 3 will result in a lack of day 21 mineralization in spite of the presence of malonate from day 3 to day 12. Interestingly β-glycerophosphate was sufficient to induce mineralization on its own in the presence of malonate, but it failed to induce it if dexamethasone and ascorbate were administered on day 0 but discontinued on day 3. These results indicate that day 3 malonate can select, on day 12, for cells that are prepared to mineralize later within several days.

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