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Studies of the Levamisole Inhibitory Effect on Rat Stromal-Cell Commitment to Mineralization

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Abstract The ability of Levamisole to decrease mineralization in skeletal tissue is usually related to its effect on alkaline phosphatase (ALP). However, Levamisole is also suspected to diminish mineralization by an additiona mechanism which is unrelated to the ALP control of apatite crystal growth. To delineate the time in differentiation during which Levamisole inhibits mineralization, a tissue culture model system of bone marrow stromal cells was used. Secondary cultures of stromal cells were propagated in osteoprogenitor cell (OPC) induction medium for three weeks, followed by measurement of calcium precipitation. In situ ALP assays at pH 7.6 were also performed. When cells were cultured with 0.2 mM Levamisole for three weeks, Day 20 values of calcium precipitates were lower than in controls, but Day 20 ALP values were paradoxically higher. The correlation between calcium and ALP within each group was low. The correlation slightly improved, in uninhibited cultures, when Day 21 calcium values were matched with earlier Day 12 ALP values. This suggested the existence of a Levamisole-sensitive mechanism for mineralization inhibition effective prior to the culture's mineralization stage. To focus on this early effect on mineralization Levamisole was added to stromal cultures on different days and removed on Day 12. Levamisole decreased Day 21 mineralization when added on Days 0, 3, 5, and 7, but not when added on Day 9. The Levamisole-induced inhibition of mineralization was accompanied by an increase in Day 12 ALP specific activity, compared to controls, when added from Day 5 and thereafter. The results indicate that part of the ability of stromal cells to mineralize is determined during the first week of culture. The early inhibitory effect of Levamisole on mineralization was associated with increased Day 12 ALP activity.

Key words: osteoprogenitor cells, differentiation, alkaline phosphatase, calcium

The mineralization of skeletal tissue has been related for a long time to alkaline phosphatase (ALP) activity in bone and cartilage cells [1]. However, the mechanism whereby ALP activity exerts its effect has not been fully revealed yet [see ref. 2 for a review]. There is evidence that mineralization-related processes, like calcium accumulation in cartilage-derived matrix vesicles or calcification in organ culture, only partially depend on ALP activity [3,4]. On the other hand, existence of mineralization disturbances in congenital hypophosphatasia, a genetic deficiency in ALP expression, indicates that ALP is associated with bone mineralization [5]. Accumulation of phosphoethanolamine [6], pirydoxal phosphate [8], and pyrophosphates [7] in the serum of such individuals is indicative of their being physiological substrates of ALP. This is consistent with ALP functioning as an ectoenzyme catalyzing extracellular hydrolysis of pyrophosphate, a crystal growth inhibitor of bone apatite, and thereby regulating extracellular mineralization at the level of apatite crystallization. Mineralization of bone nodules in culture can be inhibited by Levamisole during the very last days of crystal formation [23]. This inhibition occurs by decreasing hydrolysis of the organic phosphate in the medium [24]. However, since the dextroisomer of Levamisole can inhibit mineralization without affecting ALP activity [11], Levamisole should have an additional effect on developing bone. Bone extracellular matrix maturation, also important for mineralization, takes place between days 12 and 20 of osteoblast development in culture [18] and could also be affected by Levamisole, similarly to Levamisole inhibitory effect on collagen type X gene expression in condrocytes [25]. Based on early studies which

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have shown the importance of mitochondria during chondrocyte development in preparing cells for mineralization [review in ref. 16], Levamisole may affect the commitment of such cells to mineralization. The Fos protein complex plays a role in bone development [28]; when complexed with the Jun protein it interacts with AP1 cis elements of several important genes in many tissues [review in ref 27]. Cytokines like TNF and IL-1 can affect the AP1 nuclear transcription factor by signal transduction [27]. Levamisole increases IL-1 and decreases TNF in macrophages [29]; its effect on cytokines in developing osteoblasts or marrow stromal cells has not been studied yet. Nevertheless, it suggests a path for a possible indirect effect of Levamisole on the interaction between AP1 and Fos/Jun proteins in respect to the AP1 cis elements within VDRE regions of genes which control the commitment to mineralization in bone cells. Such interaction has been postulated as a mechanism for tissue-specific gene suppression during proliferation [20]. Therefore, it is of interest to delineate the pre-ALP activity period of stroma-derived OPCs during which they are sensitive to calcification inhibitors.

In the present work we modified a marrow stroma-cell model described by Maniatopoulos et al. [9] to examine its susceptibility to drug-induced modulation of mineralization. Attention was also paid to the correlation between ALP activity and mineralization during in vitro differentiation into osteoprogenitor cells. Levamisole was used to modulate ALP activity during mineralization and also to define the differentiation periods during which stromal cells become committed to ALP activity and to mineralization.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, Levamisole, dexamethasone, ascorbate, and β-glycerophosphate were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (Grand Island, NY).

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. [9], removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stroma cells were propagated in the same maintenance medium that consisted of DMEM (Dulbeco modified Eagle's medium) supplemented with 15% fetal calf serum and antibiotics, in a humid 10% CO_2 atmosphere, at 37°C. For the experimental cultures stroma cells were removed two weeks later by trypsinization and plated in microtiter plates, 5,000 cells/well, cultured for the first three days in maintenance medium. Day 0 is considered the day in which osteoprogenitor cell (OPC) induction medium was added to the culture. 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 activity was measured in situ in microtiter plates. On Day 12, or 20 from the start of OPC induction, the 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 optical density of the hydrolyzed pNPP was measured in a multichannel spectrophotometer at 405 nm wavelength. ALP activity was expressed either as mMol/time/cell (or well), or as optical density (O.D.) units.

For experiments which 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. 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. Following O.D. measurement the substrate of pH 7.6 was replaced by 0.1 ml of pNPP in cold 0.15 M AMP buffer (2-amino 2-methyl 1-propanol), pH 10.3. In this alkaline pH the plates were incubated for 15 min at 37°C and the reaction was stopped by quickly adding 0.1 ml of cold 0.5 N NaOH in 0.1 M Tris buffer to each well. ALP activity at pH 10.3 of every individual well was plotted against

its activity at pH 7.6 to compute their linear regression.

Quantitative Cell Staining

After the ALP assay, in some of the experiments, cells were stained with methylene blue (MB) by the method of Goldman and Bar-Shavit [10]. 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. O.D. of the eluted MB was measured at 620 nm by a multichannel spectrophotometer.

Measurement of In Vitro Precipitated Calcium

After two weeks of culture in OPC stimulation medium calcium precipitates began to appear around some of the stroma cells, demonstrable by Alizarine red (and light green counter stain) 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 staining showed foci with three-dimensional layers of calcium surrounded by cells unassociated with mineral; Stained foci contained cells located above the uninvolved cells. The precipitated calcium was washed with distilled water, vacuum dried, and analyzed by X-ray dispersive spectroscopy. It showed peaks of calcium and phosphate similar to those shown by others [9]. These foci are considered cell-mediated mineralization since, in a separate work, similar foci were almost completely absent when dexamethasone and ascorbate were removed from the cultures on Day 3, in spite of the presence of β-glycerophosphate and preserved Day 12 specific ALP activity.

Determination of the Levamisole Dose

Levamisole, at 200 μM, can inhibit 75% of the ALP activity [11,17]. When used early, its dose effect (Table I) showed a nonsignificant difference between the ability of 100 and 200 μM Levamisole to inhibit mineralization and a slight but significantly lesser effect of 200 μM on cell proliferation. The cell proliferation index on Day 12 was 0.831 ± 0.042 and 0.936 ± 0.048 (n = 20) for 100 and 200 μM Levamisole, respectively. Thus, 200 μM Levamisole, lacking a nonspecific cytotoxic effect and being sufficiently inhibitory to mineralization, was suitable for studying early ALP-independent mineralization inhibition.

TABLE I. Dose Effect of Levamisole on Proliferation and Differentiation of Stromal-Cells In Vitro

	Levamisole[a] concentrations (μM)		
	20	100	200
Ca/well index[b]	0.920 ± 0.198	0.251 ± 0.048	0.317 ± 0.052
Sp ALP index[c]	1.216 ± 0.057	1.358 ± 0.060	1.327 ± 0.073
Cell no. index[d]	0.874 ± 0.029	0.831 ± 0.042	0.936 ± 0.048

[a]Levamisole added on Day 0 and removed on Day 12.
[b]Mean ± SE ratios of [Levamisole-inhibited/control] of Day 21 calcium precipitates (mineralization index).
[c]Specific ALP index represents Levamisole effect on ALP as an early (Day 12) differentiation marker relative to controls
[d]Levamisole effect on proliferation relative to controls.

RESULTS

Correlation Between In Situ ALP Activities at pH 10.3 and 7.6

Following ALP activity and subsequently also mineralization in the same stimulated stroma-cell population, during proliferation and differention, required usage of an in situ ALP assay that is not toxic to the cells. To reassure that the in situ assay at pH 7.6 reflects values attainable at alkaline pH, a preliminary test was performed for multiple cell populations at both pH conditions. For this purpose rat bone marrow stroma cells were cultivated in microtiter wells in OPC induction medium. On Day 0 Levamisole was added to the culture to inhibit ALP activity of OPC during cell proliferation. On Day 12 cells were examined for ALP activity using the two protocols for each plate. The phosphatase activity at pH 7.6 of each well was plotted against its activity at pH 10.3. Figure 1A shows the linear regression for cells grown in OPC inductive medium; there is a direct correlation ($R^2 = 0.704$) between both phosphatase assays. Figure 1B shows the regression line of the enzyme activities in cells cultivated in the presence of Levamisole; here the correlation is even better ($R^2 = 0.845$). This indicates that 90 min of incubation at pH 7.6 is adequate for comparative

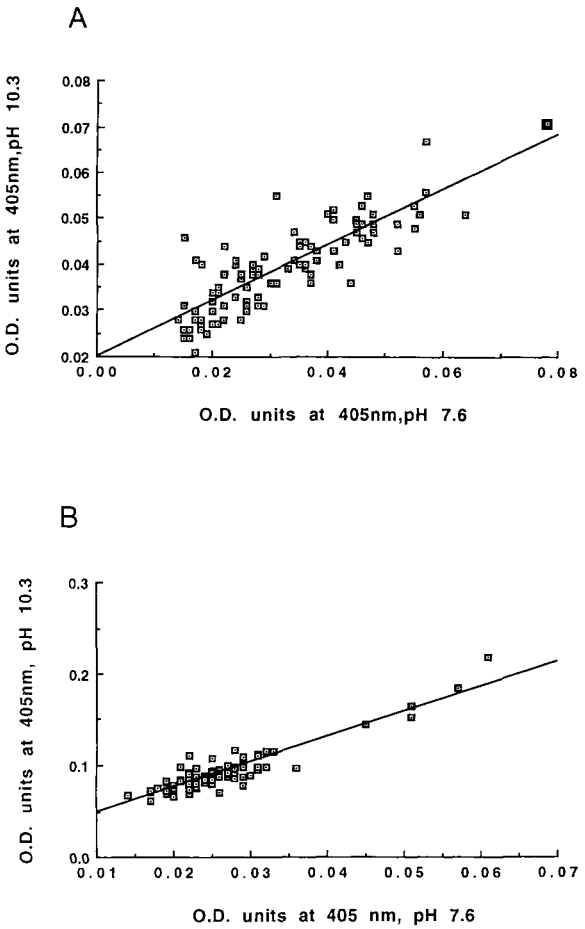

Fig. 1. Correlations between ALP activity at pH 7.6 and 10.3 in Levamizole-treated and untreated stroma cells. Stromal cells, seeded 5,000 cells/well in microtiter wells, were cultured in the absence (A) and presence (B) of 0.2 mM Levamisole from Day 0. Day 0 is determined by replacement of the growth medium by OPC stimulation medium. Correlation coefficients are R^2 = 0.704 and 0.845 in A and B.

ALP assays if groups with multiple samples are being compared. Interestingly, these results show that in cultures grown in Levamisole-containing medium the ALP activity at pH 10.3 was much stronger than the activity measured at pH 7.6.

Correlation Between Levamisole Effects on Day 20 Mineralization and ALP Activity

Bone marrow cells cultured in OPC induction medium with and without 0.2 mM Levamisole added on Day 0 were examined for ALP activity and calcium precipitation on Day 12 and Day 20. On Day 12 no calcium precipitates were detected, in spite of the ALP activity in both cultures, with and without Levamisole. On Day 20 calcium precipitates were found and, as expected, these were significantly fewer in the

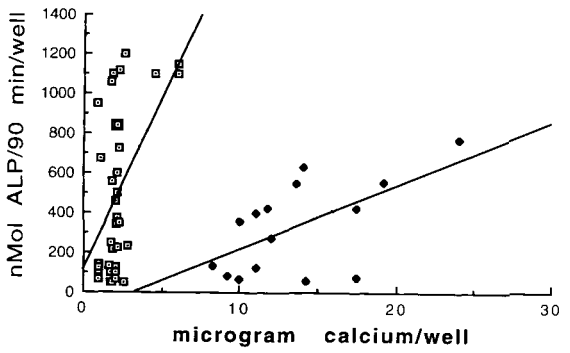

Fig. 2. Correlations between Day 21 ALP activity and calcium precipitation of Levamisole-treated and untreated stroma. Stromal cells were cultured from Day 0 with OPC induction medium in the presence (squares) and absence (diamonds) of 0.2 mM Levamisole. The correlation coefficient of Levamisole-treated and untreated cultures is R^2 = 0.252 and 0.356, respectively. The Levamisole-inhibited cultures precipitated sevenfold less calcium per unit of ALP activity than cultures without Levamisole. ALP activity in Levamisole-treated and untreated cultures was assayed in the presence and absence of Levamisole, respectively, to show the enzyme activity under these two different experimental conditions in culture.

Levamisole-treated cultures than in the untreated ones. Figure 2 illustrates two linear regressions, one of stroma cultured with and the other without Levamisole. The calcium precipitated in each well is plotted against ALP activity of the respective well. Both regression lines represent low-level correlations, R^2 = 0.252 and 0.356 for cultures with and without Levamisole, respectively. In order to test ALP activity under authentic culture conditions the ALP assay of the Levamisole-inhibited cultures was carried out in the presence of Levamisole in the pNPP substrate solution. Figure 2 shows that the main inhibitory effect of Levamisole on calcium precipitation does not parallel ALP activity on Day 20. Moreover, instead of becoming inhibited ALP activity was higher than in controls.

Correlation Between Day 21 Calcium Precipitates and Day 12 ALP Activity

To explore the possibility that Day 21 calcium precipitates may correlate with earlier ALP activity, Day 12 ALP activity was plotted against Day 21 calcium from each individual well. This experiment was performed in dexamethasone-stimulated stroma, in the absence of Levamisole. Figure 3 shows the regression line computed from Day 21 calcium and Day 12 ALP activity measurements of identical wells. The linear regression (Fig. 3) showed a slightly improved correlation between calcium and ALP,

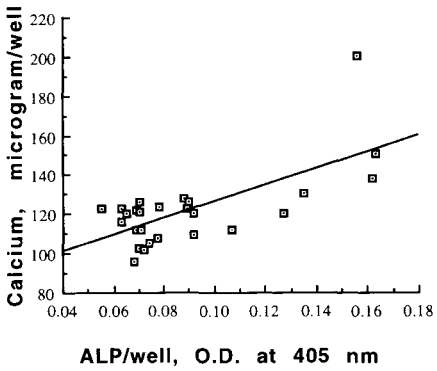

Fig. 3. Correlation between Day 21 calcium precipitates and Day 12 ALP activity in uninhibited stroma. Stromal cells stimulated with OPC induction medium in microtiter wells were analyzed for in situ ALP activity on Day 12. Subsequently stimulation continued up to Day 21, when calcium precipitates were measured. Linear regression was computed between couples of data from identical wells. $R^2 = 0.455$ (compare to 0.356 in Fig. 2), n = 26.

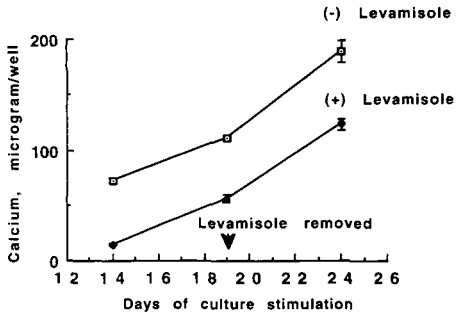

Fig. 4. Separation of the late ALP-controlled mineralization component from total, early, and late Levamisole inhibitory effect. Stromal cells were grown in OPC induction medium in the presence of 0.2 mM Levamisole. Calcium precipitates were measured on Days 14, 19, and 24 in both inhibited and control cultures. The Levamisole-containing medium in the inhibited cultures was replaced on Day 19 by regular OPC induction medium in which calcium was measured on Day 24.

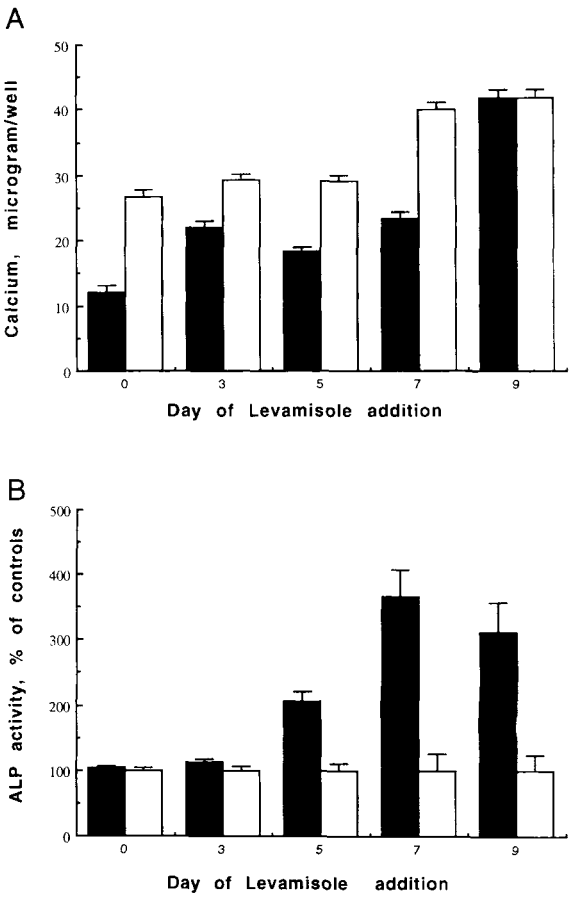

Fig. 5. Early effect of Levamisole on stromal commitment as reflected in Day 21 mineralization and Day 12 ALP activity. Levamisole (black bars) was added to two sets of stromal cell cultures during different time intervals. In one set (A) calcium precipitates were quantified on Day 21. Two sets were assayed on Day 12 (B), one for in situ ALP activity and another, stained with methylene blue, for cell quantitation. In each sample, n = 30, results are expressed as the % of specific ALP activity (per arbitrary unit of cells) relative to the respective uninhibited control's (100%) activity.

$R^2 = 0.455$ compared to 0.354 of the uninhibited culture from Figure 2.

Effect of Levamisole Applied for 18 Days on Day 24 Calcium Precipitation

To determine whether Levamisole inhibits mineralization of stroma at the precipitation level, precipitated calcium was measured on Days 14, 19, and 24 from the start of OPC stimulation. On Day 19 Levamisole was removed from the inhibited cultures. Figure 4 presents the quantity of precipitated calcium per well as a function of time. It shows that, in the Levamisole-inhibited cultures, after Levamisole was removed calcium precipitation on Day 24 did not augment to the level of the control group, but continued to increase steadily in parallel to the control curve.

Delineation of the Early Effect of Levamisole on Stroma Mineralization

To determine when the stromal cells become committed to mineralization, Levamisole, 0.2 mM, was added to cultures on different days following the addition of OPC stimulation medium. Figure 5A shows the Levamisole effect on Day 21 mineralization as a function of the time of its addition to the stroma. Calcium precipitates were diminished, relative to controls, starting from Days 0, 3, 5, and 7, but were unaffected in cultures where Levamisole was added on Day 9. Figure 5B shows the Levamisole effect on Day 12 ALP activities in relation to the time of its

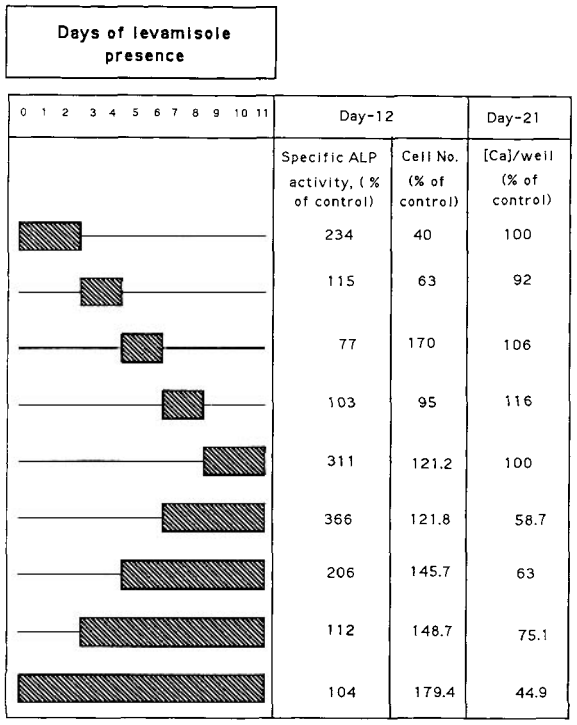

Fig. 6. Effect of short and long exposure of stromal cells to Levamisole on Day 12 specific ALP, cell number, and Day 21 calcium. Stromal cells were grown in OPC stimulation medium from Day 0 in microtiter plates. The medium in the right half of each plate was adjusted to 0.2 mM Levamisole at a different day and removed (only from the first four plates) on different days. The left half of the plates served as controls. On Day 12 ALP activity was measured at the upper two quarters of the plates and methylene blue staining for cell count was performed at the lower two quarters of the plates. A separate set of plates was used for calcium precipitation assays on Day 21. Hatched bars represent drug exposure, n = 20.

addition to the cultures. Levamisole did not induce differences of specific ALP activity between treated and control cultures when added on Days 0 and 3, but ALP activity was two- to threefold higher than the controls when Levamisole was applied from Day 5 and thereafter. Thus, Day 21 mineralization was affected by Levamisole differently then Day 12 ALP as Day 12 ALP activity, relative to controls, is either reciprocal (Days 5 and 7) or unrelated (Days 0, 3, and 9) to the Levamisole-induced differences in Day 21 mineralization. These results indicate that early commitment of stromal cells to mineralization is Levamisole sensitive and that the early drug effect on ALP activity cannot explain ALP-dependent diminished mineralization. Figure 6 illustrates different Levamisole initiation points for short time exposures, compared with different Levamisole initiation points where all exposures terminated on Day 12. For the first five groups in Figure 6 the proper regression was polynomial ($R^2 = 0.998$); for the rest the regression was linear (not shown). Day 21 calcium indices do not correlate, as a group, with specific ALP or cell count indices. Strong mineralization inhibition occurred at the long exposures to Levamisole; short exposures had a slight or no effect on calcium.

DISCUSSION

In the present experiments the effect of Levamisole on mineralization of stroma-derived OPC cultures, during differentiation in vitro, was examined.

Calcium precipitates in our experiments appear after 14 days in culture but become prominent on Day 20. Although the quantity of calcium precipitates was directly related to ALP activity when both were measured on Day 20, the correlation coefficient was low (Fig. 2). This could be due to several reasons. First, the mineralization detected on Day 20 may result from the ALP activity expressed by the cells in an earlier stage, for example on Day 12 (as shown in Fig. 3). Day 12 ALP activity correlates better with Day 20 mineralized calcium apparently because ALP and calcium reach one-third of their maximal level on Days 12 and 20, respectively, based on a projection made from Figure 1 of reference [19]. The differences between ALP/mineral correlations of uninhibited cultures in Figures 2 and 3 may indicate that ALP activity on Day 12 contributes to mineralization indirectly, perhaps by preparing the matrix or the cells for the crystallization process. Day 12 ALP was measured in subsequent experiments since it correlated with Day 20 mineral better than Day 20 ALP did.

Interestingly, the Levamisole-treated cultures showed high ALP activity in spite of the presence of 0.2 mM Levamisole during the assay. However, it is possible that this represents only 20% of the expected activity in the absence of the drug in the assay solution, since 0.2 mM Levamisole is known to inhibit 80% of ALP activity [11,17]. Second, ALP activity may not be the sole determinant of mineralization, as suggested by other workers [3,4]. Calcium accumulation can be inhibited by Levamisole, a noncompetitive ALP inhibitor; however, it has also been demonstrated that mineralization is inhibited by its stereoisomer, D-Tetramisole, which has no effect on ALP activity [4,11]. Third, as shown in Figure 5A, Levamisole inhibits stromal commitment to mineralization, suggesting that the cells reach the calcium precipitation stage lacking provisions that are important for mineral-

ization. In such a case even excessive ALP activity might fail to achieve efficient mineralization.

In our experiment in which marrow stromal cells were cultured in the presence of Levamisole from Day 0, mineralization measured on Day 20 was inhibited while ALP activity was increased (Figs. 2, 5, 6). This could reflect a compensatory reaction of the cells to the inhibited enzyme activity, by increasing ALP synthesis per cell.

Increased ALP activity under Levamisole influence probably reflects a developmental effect of this drug on OPCs. The mechanism of the *developmental* effect of Levamisole could be an inhibitory effect on expression or activity of c-fos and c-jun gene products. These oncogenes are expressed early in osteoblast development, decreasing towards Day 7 reciprocal to the increase in ALP expression [19]. This is consistent with the hypothesis that during OPC proliferation Fos/Jun protein complexes suppress transcription of tissue-specific genes like ALP and osteocalcin [20] while enhancing or not affecting cell proliferation genes. Expression of vFos or cFos increases mitochondrial retention of rhodamine 123 [21]. In addition, a fos transformation effector gene, recently cloned [22], was shown to contain a sequence for mitochondrial protein transport. Mitochondrial functions suspected to take part in early commitment to mineralization may be the link between Levamisole-induced ALP increase on one hand and mineralization inhibition on the other, both caused via c-fos inhibition, however by two independent and different mechanisms. We found that dexamethasone-containing medium increases stromal-cell retention of rhodamine (unpublished results) and that Levamisole has decreased rhodamine retention (unpublished results). Therefore, it would be interesting to test the ability of Levamisole to decrease c-fos expression.

In cultures grown with Levamisole from Day 0, removal of the drug on Day 19 did not result in a full recovery of mineralization to the level of controls on Day 24, keeping a steady $[Ca^{2+}]$gap. It is suggested that this gap (Fig. 4) represents the early inhibitory effect of Levamisole on later mineralization. Inhibition of an early mechanism probably occurred before the bulk of extracellular calcium precipitation was about to take place. Conversely, the rise in calcium precipitation after removal of the drug is probably representing the late ALP-dependent mineralization component, separated from the maturation components inhibited earlier by Levamisole. The results in Figure 4 suggest that only part of the effect of Levamisole on mineralization reflects inhibited ALP-dependent mineral deposition. The remainder of the Levamisole effect can be attributed to an earlier effect during stromal differentiation. This is consistent with the results of Register and Wuthier [3], who showed that inhibition of ALP activity in condrocytic vesicles did not inhibit hydroxyapatite crystal growth per se, it only inhibited calcium and phosphate intake by the vesicles.

Levamisole inhibited Day 21 mineralization by acting during stromal cell differentiation on Days 0 to 7 (Fig. 5A). Levamisole was not cytotoxic to stromal OPCs since Day 13 cell counts and specific ALP activity were higher than in the controls. This indicates that the Levamisole inhibition of mineralization was due to a direct cellular effect on OPCs rather than due to negative selection of non-OPCs. Levamisole has been shown to inhibit succinate dehydrogenase (SDH) activity [12]. Interestingly enough, this enzyme catalyzes the oxydation of succinate to fumarate in mitochondria of parasites at aerobic habitats, whereas in parasites of anaerobic habitats SDH catalyzes the opposite reaction [13]. This may be connected to the anaerobic conditions that promote condrocyte differentiation in the growth plate in vivo [14] and skeletal cells in vitro [15]. On the basis of calcium phosphate accumulation in chondrocytic mitochondria [16], it is reasonable to assume the existence of a mechanism that promotes aerobic glycolysis to permit calcium influx. Later, glycolysis may become anaerobic to enable calcium eflux and exocytosis. Levamisole might inhibit mineralization by interfering with either or both of these phases.

From Figures 5 and 6 it is evident that within the early Levamisole effect on mineralization there exists more than one component. The Levamisole effect on at least one inhibitory component was unrelated to its effect on proliferation and differentiation. It is also evident that early induction of Levamisole-mediated changes in mineralization was dependent on both the timing and the length of the drug administration. The experimental samples in Figure 6 were composed of two groups: one with short and interrupted exposures to Levamisole and the second lower group of gradually prolonged exposures ending after Day 11. In both groups proliferation was inversely related to differentiation and the correlation of these parameters corroborate the expected coupling consistent with the finding of Stein et al. [18].

ALP activity in this work was measured at a very mild alkaline pH, 7.6 instead of the conventional pH of 10.3. At pH 7.6 activity was 30

times slower, therefore reaction time was prolonged. Under authentic conditions ALP activity is presumably carried out at pH levels not higher than 7.6, as high pH environments are not known to exist in vivo. These assay conditions are also more authentic due to the in situ performance without prior cell lysis, which may restrict most of the phosphatase activity to the ectoenzyme form of ALP by its cell surface catalytic site. Thus, activity of intracellular phosphatases may have been largely excluded by this assay, but experiments to substantiate this assumption were not done. It cannot be excluded that some of the pNPP hydrolysis may have been caused by membrane ATPases, although the assay was performed in the presence of a nonfavorable NaCl/KCl and Tris buffer, and nonoptimal pH, all being inhibitors of Na/K ATPase [26] but lesser inhibitors of ALP.

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