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Opposing Effects on Mitochondrial Membrane Potential by Malonate and Levamisole, Whose Effect on Cell-Mediated Mineralization Is Antagonistic

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Abstract The act of chondrocyte preparation for primary, enchondral, mineralization is associated with a decline in mitochondrial respiration toward the end of the proliferative zone and the hypertrophic zone in the growth plate. Dexamethasone (Dex)-stimulated cultures of rat marrow stroma constitute a differentiation model simulating, in its energy metabolism, chondrocyte mineralization. In this model, early inhibition of succinate dehydrogenase (SDH) enriches the culture with mineralizing cells, whereas levamisole inhibits mineralization. Dex also increases mitochondrial membrane potential in stromal cells, especially on days 7–8 of stimulation. In the present study, suicide inhibition of SDH, by nitropropionic acid (NPA), in Dex-stimulated cells showed a dose-dependent increase in day 21 mineralization; the maximal effect was induced on days 2–4 of stimulation. Mineralization under 2-day-long exposure to NPA showed a similar trend to the previously studied effect of continuous exposure to malonate applied between days 3–11. Unlike malonate, the effect of NPA required its presence in the cultures for only 2 days and resulted in higher mineralization than that seen under 8 days of malonate. NPA delineated a period, days 2/4 to 7/9, in which inhibition of succinate oxidation is necessary to augment mineralization. During this period, NPA also exhibited OPC selection capacity. Early application of levamisole, under conditions previously shown to decrease day 21 mineralization, maintained mitochondrial membrane potential at the beginning of Dex stimulation but decreased or had little effect on it during days 5–10. By contrast, malonate previously found to increase day 21 mineralization decreased the membrane potential at the beginning of Dex stimulation but increased it later on day 7, or during days 5–10. These results indicate that during osteoprogenitor differentiation, before the mineralization stage, a surge in mitochondrial inner membrane potential during late matrix maturation may be a marker that heralds the extracellular matrix mineralization.

Key words: mitochondrial membrane potential, malonate, levamisole, osteoprogenitor differentiation, extracellular matrix mineralization

Initiation of enchondral mineralization depends on calcium accumulation in maturing chondrocyts [Wuthier, 1993] via nifedipine and verapamil-inhibitable channels [Zimmermann et al., 1994], it may also depend on phosphate accumulation [Montessuit et al., 1994]. In hypertrophic chondrocytes [Posner, 1978] as well as in soft tissues [Lehninger, 1970], the mitochondria accumulates amorphous calcium phosphate in a respiration dependent manner. Mitochondria of hypertrophic chondrocytes can accumulate more calcium than that of soft tissues [Shapiro and Lee, 1975]. Although the mitochondria are not the main site for intracellular calcium storage [Carafoli, 1987; Pozzan et al., 1994], in the case of chondrocytes there is still a basis for implicating the mitochondrial calcium deposits in primary mineralization. The drop in oxygen tension [Brighton and Heppenstall, 1971; Haselgrove et al., 1993], the increased phosphoenolpyrovate [Shapiro and Lee, 1978], and the increased NADH/NAD ratio [Shapiro et al., 1983] at the hypertrophic zone are indicative of a transition from aerobic to anaerobic metabolism, in which the mitochondria plays a central role. Deposition of intramitochondrial calcium in the extracellular matrix (ECM) of the growth plate [Brighton and Hunt, 1978] also suggested involvement of this organell in the mineralization process. In soft tissues under aerobic conditions, mitochondrial accumulation and release

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of calcium are respiration dependent; however, calcium release occurs via channels different than those of calcium uptake [Lehninger et al., 1978a]. Calcium release can occure by Na^+-dependent or Na^+-independent mechanisms and also by a nonspecific way [Richter, 1992].

Most of the studies on calcium release were performed in mitochondria of soft tissues in which calcium fluxes are transient and serve as signals, whereas in hard tissues calcium also serves as an ECM mineral deposit. It is not cleare whether the same mechanisms of calcium release from mitochondria in soft tissue are used in hard tissue to initiate calcium deposition. In several soft tissues mitochondrial calcium retention is associated with a high NADH/NAD ratio, whereas calcium release in exchange for protons is associated with a low ratio [Lehninger et al., 1978b]. This ratio should depend on the respiration level, reflected by the membrane potential. Thus, the high NADH/NAD ratio found in hypertrophic chondrocytes [Shapiro et al., 1983] agrees with a low oxygen tension, and with calcium retention by the mitochondria in this zone of the growth plate. The growth plate membrane potential profile is not known. Presumably after a sustained low membrane potential at the hypertrophic zone, the membrane potential should increase prior to mineralization in front of the zone of provisional calcification. This should be expected on the basis of studies with a different osteogenic differentiation system (rat marrow stromal cells) in which a drop in membrane potential has been shown from days 3–4 of subconfluency, and maintained up to day 10. When stimulated to mineralize by dexamethasone (DEX) medium the membrane potential followed a similar pattern but with a twofold increase in membrane potential and on day 7–8 an eightfold burst in membrane potential [Klein et al., 1993c]. In this system, in which mineralization becomes detectable on day 13–14, the membrane potential surge could be a prelude to mineralization. Inhibition of succinate dehydrogenase (SDH, complex II) by malonate [Klein et al., 1993a] corroborated the finding by Shapiro et al. [1983] that cells destined to mineralize rely less on respiration. Also the efficacy of osteoprogenitor cells (OPC) selection by malonate was concurrent with the period of lower membrane potential. Malonate resulted in selection of OPC but showed also an absolute 20% increase in mineralization. Levamisole effect on mineralization is opposite to that of malonate, it is an anthelmintic drug that inhibits the fumarate reductase activity of SDH in adult helmintic parasites during their anaerobic stage [Van den Bossche and Janssen, 1969]. In rat stromal cells, levamisole was shown to inhibit mineralization by a manner unrelated to its inhibitory effect on alkaline phosphatase [Klein et al., 1993b]. In the present work, we have outlined the temporal effect of SDH inhibition on mineralization, using nitropropionic acid (NPA), which is a more effective SDH inhibitor than malonate. NPA is a suicide inhibitor of SDH since unlike malonate it becomes tightly bound to the enzyme [Coles et al., 1979]. In addition we show that two drugs, malonate and levamisole, with opposing effects on mineralization and perhaps inhibitory effect on opposite SDH activities, also have opposing effects on the profile of the mitochondrial membrane potential during OPC differentiation.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate, nitropropionic acid (NPA), and rhodamine 123 were purchased from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Grand Island Biological Company (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 [Maniatopoulos et al., 1988]. Stromal cells were obtained by removing the non adherent 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% FCS and antibiotics. The cells were incubated in a humid 9% CO_2 atmosphere, as in all our previous studies, as increased CO_2 is associated with a decreased redox level in differentiating bone cells. For the experimental cultures, stromal cells were removed 2 weeks later by trypsinization and plated in 96-well microtiter plates, 5,000 cells/well, and grown in OPC stimulation medium. This consisted of maintenance (ordinary) medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate, and 10 mM β-glycerophosphate. The medium was changed every 3–4 days.

Rhodamine Retention Measurement

Rhodamine 123 (Rho) is a cationic dye trapped by the mitochondria, depending on the content

of its negative charges, and directly related to the inner membrane potential [Chen, 1989]. Rho stock solution × 20 in distilled water was added to the cultures, 10 μl/well, after an incubation period as indicated, the Rho-containing medium was removed. The cells were washed twice with TBS (50 mM Tris pH 7.6, 150 mM NaCl) and continued their cultivation after the short incubation period until Rho retention measurement, the following day.

After removal of the growth medium and washing with TBS, the cells were incubated for 30 min at 37°C with 150 μl of 0.2% Triton-X 100 to dissolve membranes and to release intracellular Rho. The dissolved cell sap was transferred to an opaque-white 96-well plate. Rho content was measured in a Perkin Elmer LS-5B luminescence spectrometer with a plate reader, by exitation at 505 nm and emission at 540 nm.

Quantitative Cell Staining

After the ALP or the rhodamine retention assays cells at the lower half of the plates were stained using the methylene blue (MB) method. 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 optical density (O.D.) of the eluted MB was measured at 620 nm by a multichannel optical densitometer. Cells seeded at different seeding levels on day 0 (250, 500, 1,000, 2,000, and 4,000/well). On days 10 and 13, close to the usual time for cell counting in our experiments, the cells were divided into two groups; four wells for each were trypsinized and counted; in parallel, eight wells were MB stained. Linear regressions for counts and MB staining on both days 10 and 13 were practically coalescent and indicate that linearity of the O.D. reading at least up to 73,000 cells/well, 1.0 unit is equivalent to 5×10^4 stromal cells.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 of dexamethasone stimulation was set for ALP assay and cell count [Klein et al., 1993a]. Growth medium was removed, and the cells were washed twice in situ with 0.2 ml TBS (50 mM Tris, 150 mM NaCl, pH 7.6). ALP substrate, pNPP (*p*-nitrophenyl phosphate) in TBS, 1.33 mg/ml was dispensed 0.2 ml/well. Plates were placed in the tissue culture incubator for 90 min, and O.D. of the hydrolyzed pNPP was measured in a multichannel optical densitometer at 405 nm wave length. This protocol enables long incubation times as hydrolysis is 30 times slower than the usual protocol [Klein et al., 1993b]. ALP specific activity was calculated as nmol/90 min/50,000 cells and expressed as an activity index (experimental/control specific ALP activity).

Measurement of In Vitro-Precipitated Calcium

After 3 weeks in culture, OPC stimulation medium as opposed to ordinary medium induces cell-mediated calcifications in uninhibited cultures [Klein et al., 1993b]. To measure the precipitates, plates were washed twice with TBS and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations. Mineralization is expressed as μg Ca/well and presented as indices of experimental to control ratios.

RESULTS

Inhibition of Succinate Dehydrogenase

Stromal cells stimulated with Dex medium were exposed during days 0–11 to three different NPA concentrations. Figure 1 shows that a maximal effect of NPA on cell counts and specific ALP activity were obtained at 10 mM concentration. Under 10 mM NPA, the cell count was 2.5 times less (relative to the cell count under 0.1 mM NPA), to account for the respective increase in specific ALP activity. This was possibly due to a toxic effect of the high NPA concentration on ALP expressing cells. For this length of exposure (days 0–11), the appropriate dose for OPC selection is probably between 1 and 10 mM (Fig. 1A). As expected there was an inverted correlation between the change in specific ALP activity and the change in cell count (not shown), which was linear up to an NPA concentration of 1 mM and became logarithmic between 1 and 10 mM, reflecting the toxic effect of the high dose. It seemed possible that OPC sensitivity to NPA differs from one differentiation stage to another. To reveal the period of stromal differentiation during which OPCs mineralization is affected by NPA the drug was applied for shorter and different periods, permitting also to decrease undue effects on surviv-

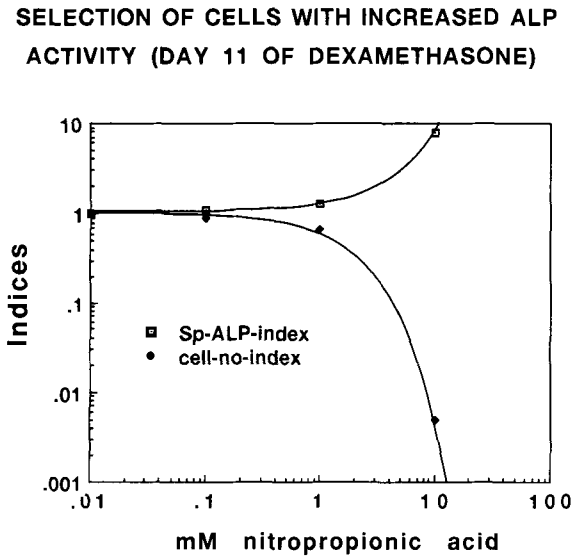

Fig. 1. Proliferation and matrix maturation stages of differentiation of the stromal OPC under suicide SDH-inhibition. Dex stimulated stromal cells were exposed to three different doses of the SDH suicide-inhibitor NPA from day 0 to 11 in the left half of the plates. Each point is the mean index between respective wells of [NPA-inhibited]/[noninhibited] specific ALP activity and cell count on day 11 (n = 20).

ing OPCs. Figure 2 shows a disection of time interval exposure to NPA, as a dose response of cell-mediated mineralization, measured on day 21 of Dex stimulation. On exposure periods, days 0–2 and days 9–11, 10 mM and 1 mM NPA has not increased the mineralization significantly; day 2–4 was the most efficient period for early induction of mineralization under all three NPA concentrations. This finding is consistent with the previous finding in which initiating of SDH inhibition on day 3, using a competitive inhibitor, showed the best OPC selection efficiency [Klein et al., 1993a]. Since the effect of malonate, a competitive SDH inhibitor, on cells is less severe than that of NPA we have later used malonate to study effects of complex-II inhibition on the profile of mitochondrial membrane potential.

Short Time Effect of Levamisole on Rho Retention

We have previously shown that levamisole has an early inhibitory effect on mineralization which is unrelated to its inhibitory effect on ALP [Klein et al., 1993b]. Figure 3 shows the effect of levamisole, added at different days during OPC differentiation, on Rho retention 24 hr after its application. It showed a general tendency to increase Rho retention at the first 4 days of Dex stimulation (Fig. 3A) regardless of and perhaps even reciprocal to cell proliferation (Fig. 3B). This is better demonstrated by the indices in Figure 3C, showing that the peak of increased Rho retention was on days 2–3, compared to the Dex controls. From day 4 to day 10,

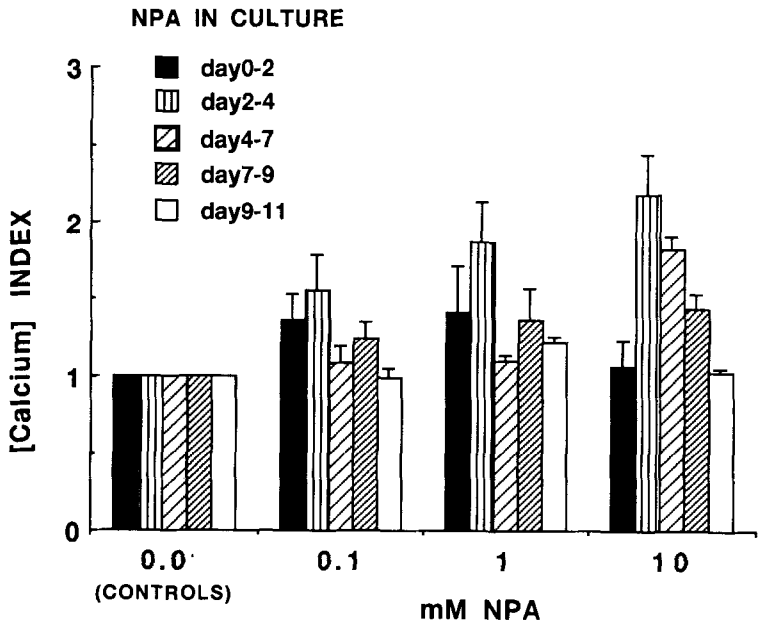

Fig. 2. OPC mineralization dose response and temporal disection by NPA administration at various time intervals. Mean ±SE of the change in mineralization ([calcium] index) induced by NPA relative to control cultures. All cultures were stimulated by Dex medium from day 0 to 21, insoluble calcium was measured on day 21, n = 20 wells/sample.

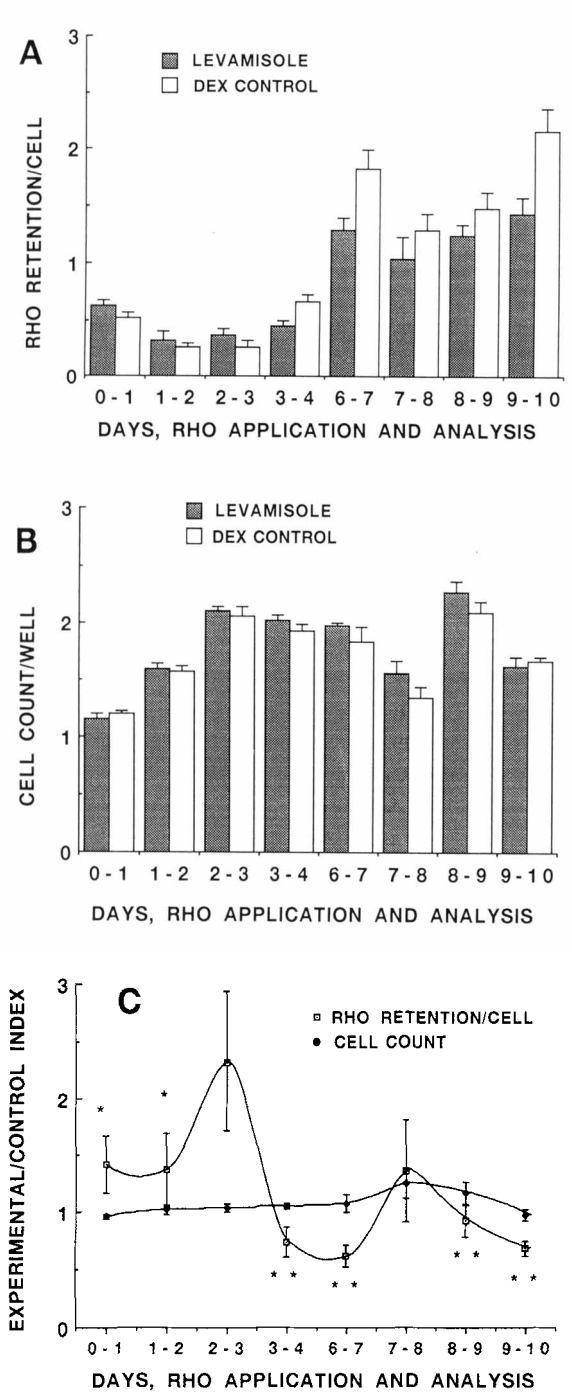

Fig. 3. Disection of the short term levamisole effects on mitochondrial Rho retention in Dex stimulated stromal cell cultures. Stromal cells were Dex-stimulated on day 0 in 8 separate plates. Rho, 0.1 μg/ml, was added to experimental and control cultures and levamisole (0.2 mM final concentration) was added to experimental cultures on different time intervals as indicated. After 24 hr, the respective cultures were washed twice with TBS and Rho-extracted with 0.15 ml 0.2% Triton X-100 dissolved in distilled water at 37°C for 30 min. **A:** Rho fluorescence/cell quantity. **B:** Cell quantity. **C:** Levamisole effect on Rho retention and cell quantity relative to controls, n = 10 wells/point. Significance by which day 2–3 differed from the rest: $*\alpha < 0.05$ for one-tailed, $**\alpha > 0.05$ for two-tailed test by the Mann-Whitney U-test.

levamisole diminished Rho retention compared to Dex controls, except on days 7–8, when it was slightly higher but not significantly different from unity. The peak of mean Rho retention on day 3 was significantly higher, more than two-fold, of that in Dex controls. On days 7–8 levamisole seemed to have failed to diminish Rho retention, which raises under Dex [Klein et al., 1993C].

Cumulative Effect of Malonate on Rho Retention

Figure 4A and 4C show that malonate diminished the cell counts after 7 days of application. The basic pattern of Rho retention under Dex stimulation is shown by the controls (Fig. 4B). Malonate increased Rho retention on day 7. The question was raised as to whether, under the same protocol in which it inhibited mineralization [Klein et al., 1993b], levamisole would show an effect on Rho retention reciprocal to the pattern seen in Figure 4C.

Cumulative Effect of Levamisole on Rho Retention

Figure 5A and 5C show that levamisole diminished cell counts on day 1 and similarly to malonate also on day 7. Levamisole has slightly increased cell counts on day 10, which was not a surprise, since it was expected to increase on day 11 or 12 [Klein et al., 1993b]. Under prolonged exposure, levamisole diminished Rho retention from days 3 to 10 (Fig. 5B), in contrast to the effect of malonate seen in Figure 4B and 4C.

Combined Effect of Malonate and Levamisole on Rho Retention

Figure 6 shows the Rho retention/cell at different time intervals after exposure to both, malonate and levamisole in the same Dex-stimulated cultures. Here the separate effects of each drug serve as controls for the effects of their mixture. Levamisole succeeded in inhibiting Rho retention in spite of the expected augmenting effect of malonate, and only on day 10 was the levamisole inhibitory effect on Rho retention completely abolished by malonate.

DISCUSSION

In the present study, prolonged exposure of stromal cell cultures to 10 mM NPA caused an abrupt fall in cell number accompanied by an increase in specific ALP, which was less than expected for a mere selection effect. This suggested that OPC at a certain stage return to

depend on SDH or perhaps its utilization is never completely shutdown as suggested by Pollesello et al. [1991], who demonstrated low-level oxidative respiration activity in hypertrophic chondrocytes. In addition, complex II in OPC may exhibit a dual function in accord with the transition from aerobic to anaerobic respiration. This has been shown in vitro. Using a direct voltametric method, Sucheta et al. [1992] have shown that SDH of bovine heart mitochondria behaves as a diode; i.e., the switch from succinate oxidation to fumarate reduction (performed by the same enzyme, complex II) depends on the raise or decline of the electrochemical potential, respectively. As a suicide SDH inhibitor [Coles et al., 1979], NPA would induce cell death whenever demand for SDH activity will rise to a very minimal level. Therefore, at 10 mM NPA its stringent effect on complex II should not allow redox activity in either direction of this putative diode under any electrochemical potential. Probably, either a part or most of the ALP expressing cells may require some SDH activity, such that the observed anaerobic period might not be absolutely anaerobic. This may explain why in a previous study malonate, which is only a competitive SDH inhibitor, was less effective than NPA in diminishing the cell counts relative to the increased ALP activity/cell [Klein et al., 1993a]. The use of NPA at short intervals clearly showed that decreased SDH activity positively affects the preparation of differentiating OPC to mineralize, in addition to its ability to act like malonate by enrichment of OPC in culture. A physiological association of cell death with mineralization, possibly programmed cell death, can not be dismissed. Interestingly, the highest increase in day 21 mineralization occurred upon day 2–4 exposure to NPA, a period after which mitochondrial membrane potential becomes resistant to changes induced by prolyl hydroxylase inhibition with cis-hydroxyproline (cis-HP) [Klein et al., 1994]. Cis-HP inhibits prolyl hydroxylase [Kivirikko and Myllyla, 1987]. Hypothetically, inhibition of prolyl hydroxylase should prevent

Fig. 4. Cumulative effect of malonate on Rho retention. Differentiating stromal cells, Dex stimulated from day 0, underwent medium adjustment to 10 mM malonate on day 0. Mitochondria in malonate-treated and control cultures was loaded with Rho, 24 hr before the indicated days, by incubation with 1 μg/ml for 30 min at 37°C. Excess Rho was removed by washing the cultures twice with TBS at room temperature and cultivation was continued in the appropriate media. Rho was extracted 24 hr later and measured as described in the legend of Figure 3; cell counts were obtained from nearby wells. **A:** Effect of malonate on cell count. **B:** Malonate effect on specific Rho retention. **C:** Malonate effects on cell count and on Rho retention relative to untreated controls, n = 10/sample.

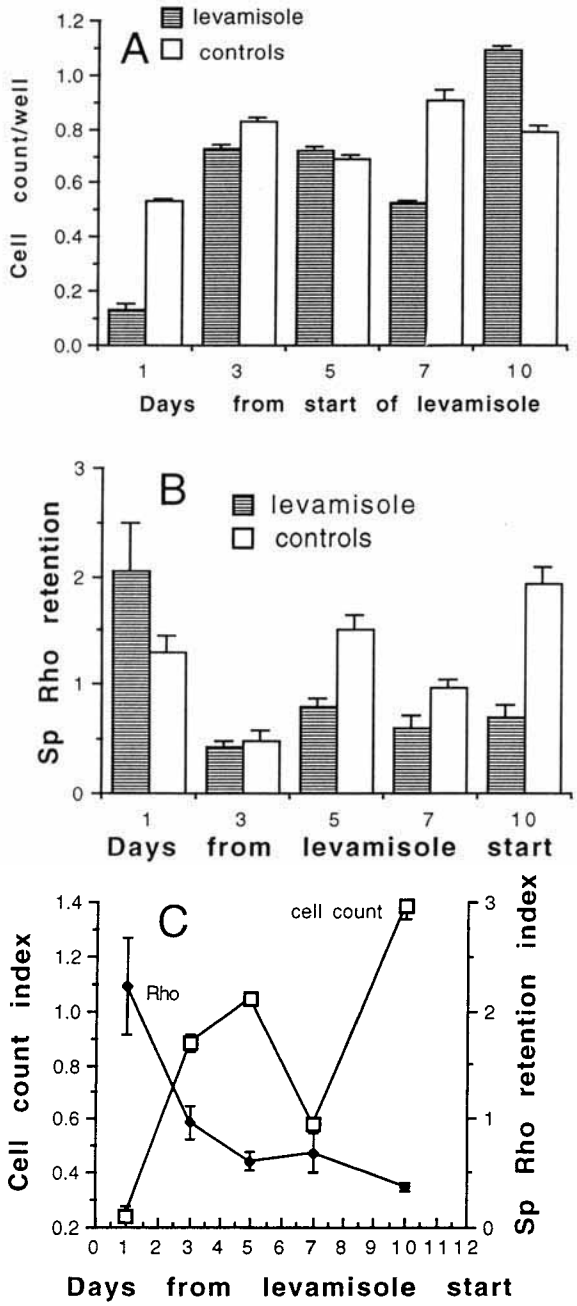

Fig. 5. Cumulative effect of levamisole on Rho retention. The same protocole as described in the legend of Figure 4, with the exception that on day 0 the medium in the experimental cultures was adjusted to 0.2 mM levamisole.

free oxygen use by the endoplasmic reticulum and increase oxygen use by mitochondria, which in turn should increase mitochondrial membrane potential. This was indeed shown to be the case in a previous study however, only up to day 5 of Dex stimulation, when membrane potential became resistant to prolyl hydroxylase inhibition [Klein et al., 1994]. It should be noted that the temporal dissection of the NPA effect on mineralization has discerned a period (days 2–4/7–9), which is consistent with the decline in membrane potential in non stimulated stromal cultures [Klein et al., 1993c].

The initiation of mineralization can be perceived as a calcium and phosphate efflux from the mitochondria directly to the extracellular matrix (ECM), serving as a primary crystalization nidus or as a signal, together with phosphoproteins [Wuthier, 1993], to from membrane-derived ECM vesicles for initiating crystalization, or both.

The different, perhaps opposing, effects of these two drugs (levamisole and malonate) on mineralization are attributable to the ability of levamisole to abolish the Dex-induced increase in membrane potential after 3 days in culture, as opposed to the ability of malonate to abolish it on the first days and increase it thereafter.

Under the specified conditions levamisole can resist the effect of malonate, on the Dex-induced Rho retention, during most of the matrix maturation period, which is tentatively defined as day 3–12. Only on day 10 levamisole has lost the ability to resist the effect of malonate on Dex-induced Rho retention. The dominance of the levamisole effect over that of malonate may be due to the differences in the mechanisms by which they inhibit complex II, noncompetitive versus competitive inhibition respectively.

We conclude that modulation of the membrane potential during the first differentiation period (matrix maturation) and the mineralization taking place later at the second period, represent a cause-and-effect relationship. Thus the inner mitochondrial membrane potential is a target function through which mineralization is modulated long before it is actually carried out. The accumulated succinate during inhibition of SDH could be stockpiled to serve later as an electron source for a surge in membrane potential and for securing return of expelled protons in exchange for calcium efflux. Accordingly, the mineralization improving effect of malonate and NPA could be carried out by further augmenting an already existent Dex-induced surge of day 7–8 membrane potential.

In light of these results, one should look into the biochemical meaning of the ability of levamisole and malonate (mineralization inhibitor and inducer, respectively) to affect the Dex-induced mitochondrial Rho retention. In light of the present results, the parallel phenomena of aerobic/anaerobic transition in helmintic parasites

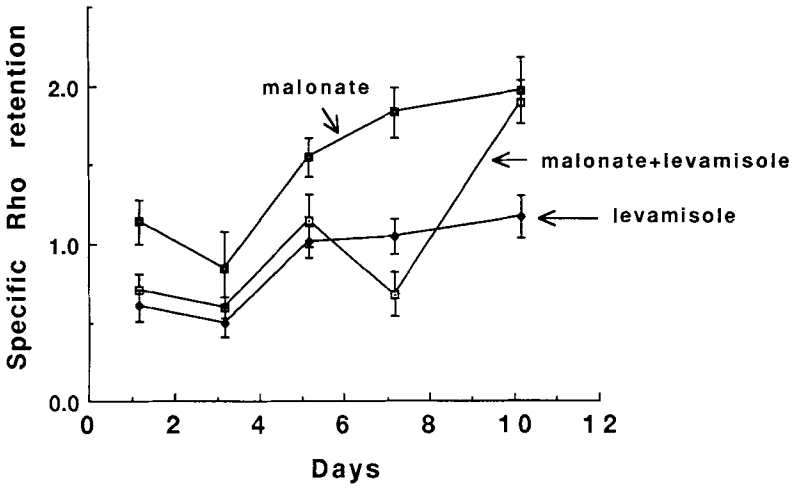

Fig. 6. Cumulative effect of malonate combined with levamisole on Rho retention during OPC differentiation. The medium of stromal cells that were Dex-stimulated on day 0 was adjusted on day 0 to a concentration of 10 mM malonate and 0.2 mM levamisole. Two controls were used, each treated with only one of the two drugs. Analysis of Rho retention was performed as described in the legend of Figure 4, each point is the mean ±SE of 10 replicas.

[Roos and Tielens, 1994] and in osteogenic stages, and the involvement of complex II in this transition and its ability in vitro to switch catalytic directions [Sucheta et al., 1992], raise several questions. Does complex II in differentiating OPC act as a diode, switching catalytic directions?

Does levamisole-inhibited mineralization also mean that fumarate reductase activity has a positive role in mineralization? Are there extramitochondrial macromolecules that regulate this phenomenon? Is it possible that the pattern of membrane potential determines the timing of mitochondrial calcium and phosphate fluxes to provide an appropriate stoichiometry in generating nidii for apatite crystal growth?

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