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Analysis of Cell-Mediated Mineralization in Culture of Bone-Derived Embryonic Cells With Neurofibromatosis

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Abstract von Recklinghausen neurofibromatosis (NF1) is an autosomal dominant genetic disorder associated with congenital pseudoarthrosis and with short stature. To examine whether the NF1 phenotype includes functional osteogenic defects, embryonic bone-derived cells affected with NF1 were tested in culture for specific alkaline phosphatase (ALP) activity and cell-mediated mineralization and compared with other embryonic bone derived cells. NF1 showed a relatively higher specific ALP activity, which has further increased in response to dexamethasone + β-glycerophosphate (βGP) (Dex medium) coordinately with a decrease in cell proliferation. In the control group, two samples showed increased ALP activity, one showed decreased activity and the forth one did not show any change in ALP. NF1 cells were distinguished from other cells regarding day 21 mineralization, they did not mineralize when cultured with ascorbate alone in the abscence of Dex medium, whereas control cells did mineralize. Adding βGP resulted in mineralization by NF1 cells but less than in other cells. In addition, NF1 cells responded to dexamethasone by increasing the βGP-induced mineralization, as opposed to cells from other embryonic bones, which either did not respond or have even decreased mineralization under dexamethasone. Upon cis-hydroxyproline exposure, Dex medium has also distinguished NF1 cell ALP activity from that of other cell origins. Inhibition of respiratory complex II by malonate showed that most embryonic bone-derived cells of 12 weeks gestation are malonate resistant; thus, malonate selection was ineffective. This is in contrast to rat marrow stromal cells previously shown to undergo mineralizing cell enrichment in response to malonate. Exposure to levamisole, of Dex-treated cells, at days 0–11 has inhibited day 21 mineralization in all tested cultures in spite of the increase in day 11-specific ALP activity. Both malonate and levamisole did not distinguish NF1 cells from the osteogenic phenotype of other cells. Essentially embryonic bone-derived cells from 12 weeks gestation, cultured in the absence of βGP, retained their mineralization capacity, which does not increase under dexamethasone, as distinguished from NF1 cells which require βGP for mineralization and positively respond to dexamethasone. Therefore, bone-derived NF1 cells may be useful for studying the regulation of the mineralization process.

Key words: alkaline phosphatase, β-plycerophosphate, dexamethasone, malonate, levamisole, cis-hydroxyproline

The normal structure of calcified skeletal tissues probably depends on a mineralization process coordinated with a properly organized construction of extracellular matrix (ECM). The route through which various chemical groups and minerals join the bone apatite during its growth is not yet fully understood. However, it is believed that cellular mechanisms are responsible for generating the first apatite crystal nidus as a template for crystal growth. Part of the processes responsible for committing skeletal cells to mineralize are known. For example, it has long been known that in the growth plate chondrocytes, reaching their hypertrophic stage, temporarily enter a hypoxic phase [Brighton and Heppenstall, 1971]. This is associated with the restricted preference of the glycolytic pathway for the cell's energy metabolism [Shapiro et al., 1982] and with a decrease in mitochondrial respiration. The hypoxic state is also associate with accumulation of calcium phosphate in the mitochondria of these chondrocytes [Lehninger, 1970]. Part of this calcium phosphate reaches the ECM at the provisional mineralization zone [Brighton and Hunt, 1978]. This hypoxic state is probably subjected to regulation within defined

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oxygen concentrations, since excessively low or high oxygen tension in the culture was show to decrease the osteogenic effect [Deren et al., 1990]. We have shown that dexamethasone stimulation of stromal cells, which induces their mineralization in culture, has also increased cell respiration [Klein et al., 1993c], as indicated by mitochondrial retention of rhodamine 123. The *fos* oncogene that is a component of the AP1 complex and is involved in osteogenesis [De Togni et al., 1988] also increases mitochondrial rhodamine retention [Zarbl et al., 1987]. It is therefore possible that commitment to mineralization during differentiation is regulated via a pathway that includes a c-*fos* activity that influences mitochondrial respiration. One way to test this hypothesis is to analyse mineralization of bone cells from a source deficient in a gene functionaly related to c-*fos* activity and also with mineralization-related bone defects. von Recklinghausen neurofibromatosis (NF1) is such a disease. It includes among multiple manifestations several orthopedic problems [reviewed by Riccardi, 1981] of which short stature and pseudoarthrosis are related to epiphyseal growth and possibly to mineralization disturbance. The NF1 is an autosomal dominant genetic disorder in which the normal neurofibromin (NF1 gene product) is inactive [reviewed by Viskochil et al., 1993]. Neurofibromin containes a defined sequence homology to the GAP (GTPase activating protein) of *ras*. In spite of the GAP domain contained in the NF1 gene, it is not known at which stage, if at all, neurofibromin reacts with *ras*. Since signal transduction via the *ras* pathway activates c-*fos* it is reasonable that, normally, if and when neurofibromin reaction with *ras* is required but not carried out due to neurofibromin inactivity, the effect of c-*fos* on mineralization will be hampered. In the present study, embryonal cells were tested for the ability to retain ALP activity and mineralization capacity in culture, under conditions adapted from rat stromal cell studies. We present results which distinguish the mineralization capacity of NF1 embryonic bone cells from non-NF1 embryonic bone cells.

MATERIALS AND METHODS

Reagents

Cis- and trans-hydroxyproline, ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate, and sodium malonate were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Grand Island Biological Company (Grand Island, NY).

Embryonic Bone-Derived Cell Culture

Bone samples obtained under informed concent were resected from femora tibiae and ribs of human fetuses at 12–13 weeks gestation. The cell populations from the individual origins were designated EmL and were sequentially numbered. The gestations were terminated due to social (EmL-3 and EmL-4), genetic [neurofibromatosis designated EmL-1 (NF1), Gaucher disease, designated EmL-5 (GD)], and pathologic [hepatitis C, designated EmL-2 (HCV)] reasons. Bone samples cut in 1- to 2-mm pieces and placed in 60-mm tissue culture dishes were cultured in DMEM (Dulbecco modified Eagle's medium) supplemented with 15% fetal bovine serum (FBS) and antibiotics, in a humid 9% CO_2 atmosphere. After 10–15 days, upon confluence, cells were trypsinized and transferred to flasks. Part of the cells were frozen for later use or directly seeded in 96-well microtiter plates, 10,000 cells/cm^2. The cultures were grown in osteoprogenitor cell (OPC) stimulation medium (DEX medium) or in DMEM with 10% FBS (ordinary medium) with variations specified in the text. The Dex medium consisted of ordinary medium containing 10^{-8} M dexamethasone, 50 μg/ml ascorbate, and 10 mM β-glycerophosphate, variations are specified in the text. The medium was changed every 3–4 days.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was measured in situ in microtiter plates. Day 11 or 12 of dexamethasone stimulation was set for ALP assay as described before [Klein et al., 1993b]. Briefly, after medium removal 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 a tissue culture incubator for 90 min, and the optical density (O.D.) of the hydrolysed pNPP was measured in a multichannel spectrophotometer at 405-nm wavelength.

ALP specific activity was expressed either as O.D. or nMol/time/cell quantity, or as an activity index or percentage of control (experimental/control-specific ALP ratio). To obtain experimental/control indices and ALP activity/cell

quantity, the results of individual wells were divided by those of the corresponding wells at the control side of the plate. This was done taking into account the position uniqueness of each well within every quarter in the microtiter plate, regarding variations in physical conditions like temperature and gas exchange. According to this, any result of well A2, for example, can be related only to that of A11 or H2 or H11. This practice can correct also for putative effects derived from temperature and gaseous interactions between wells within the same plate, a phenomenon previously observed in this system [Klein et al., 1994].

Quantitative Cell Proliferation

After the ALP or the rhodamine retention assay cells at the lower half of the plates were stained using the methylene blue (MB) method for cell quantitation [Goldman and Bar-Shavit, 1979]. 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 cells.

Measurement of Precipitated Calcium in Culture

To quantitate calcium precipitates plates were washed twice with TBS and with distilled water and incubated in 0.5 N HCl overnight. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations or by the colorimetric method in which calcium binding to the dye Arsenazo III changes its optical density. The method described by Bauer [1981] was modified adjusting it to our culture system. Samples for the Arsenazo III assay consisted of 10 μl of the acid dissolved calcium from each well and 190 μl of 0.85 M Arsenazo, dissolved in TBS (pH 7.6) to buffer the calcium sample acidity. A standard curve of $CaCl_2$ was set for each plate and consisted of serial dilutions of a 2.0 μg calcium equivalent. In addition, samples of known calcium concentrations were placed in each plate to control for accuracy of the test. After 30-min incubation, all the samples were diluted 1:5, and 200-μl aliquots were transferred to a clean plate. The O.D., at 620 nm, was recorded, and calcium concentrations were computed using a multichannel densitometer with a plate reader. The final result of calcium equivalent/well was obtained after correction for the dilution factor. Figure 1 shows the correlation between calcium measurements of the same samples by atomic absorption and by Arsenazo III binding

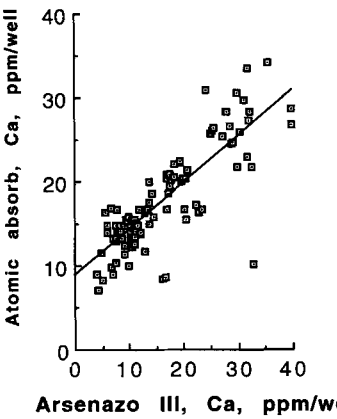

Fig. 1. Correlation between two calcium assays: atomic absoption and Arsenazo III binding. Equal volumes of HCl-dissolved calcium samples were measured for calcium concentration by both methods. The correlation coefficient of the presented linear regression is r = 0.820, n = 99.

(r = 0.820). In the present study, we used 40 replica per sample for calcium measurements in most of the experiments.

The precipitates were expressed as μg Ca/well or presented as indices of experimental to control ratios.

RESULTS

ALP Activity and Proliferation Between Days 5 and 11

Figure 2 shows the effect of DEX medium on ALP activity in embryonic bone cells of different individuals, the cells in these experiments were from secondary cultures. Under DEX stimulation, the specific ALP activity in three cultures increased above the unstimulated controls, the absolute ALP activity of EmL-1(NF1) was at least twofold higher than that of EmL-2(HCV), EmL-3(normal), and EmL-5(GD) during the 11-day stimulation. The "normal" EmL-4 cells did not respond to Dex medium on day 5 and responded by decreasing ALP activity on days 8 and 10. The ALP activity in EmL-5(GD) cells did not change in response to Dex medium. EmL-1(NF1) responded early to DEX stimulation, it has increased twofold relative to unstimulated controls (Fig. 2B) showing a slight decline on day 11, whereas in EmL-2(HCV) and EmL-3(normal), the main response was detected only between days 7–9 to day 11, in which the ALP index rose close to twofold.

Figure 3 shows the cell proliferation response to DEX stimulation. The proliferation trend (Figure 3A and 3B) was reciprocal to that of the ALP

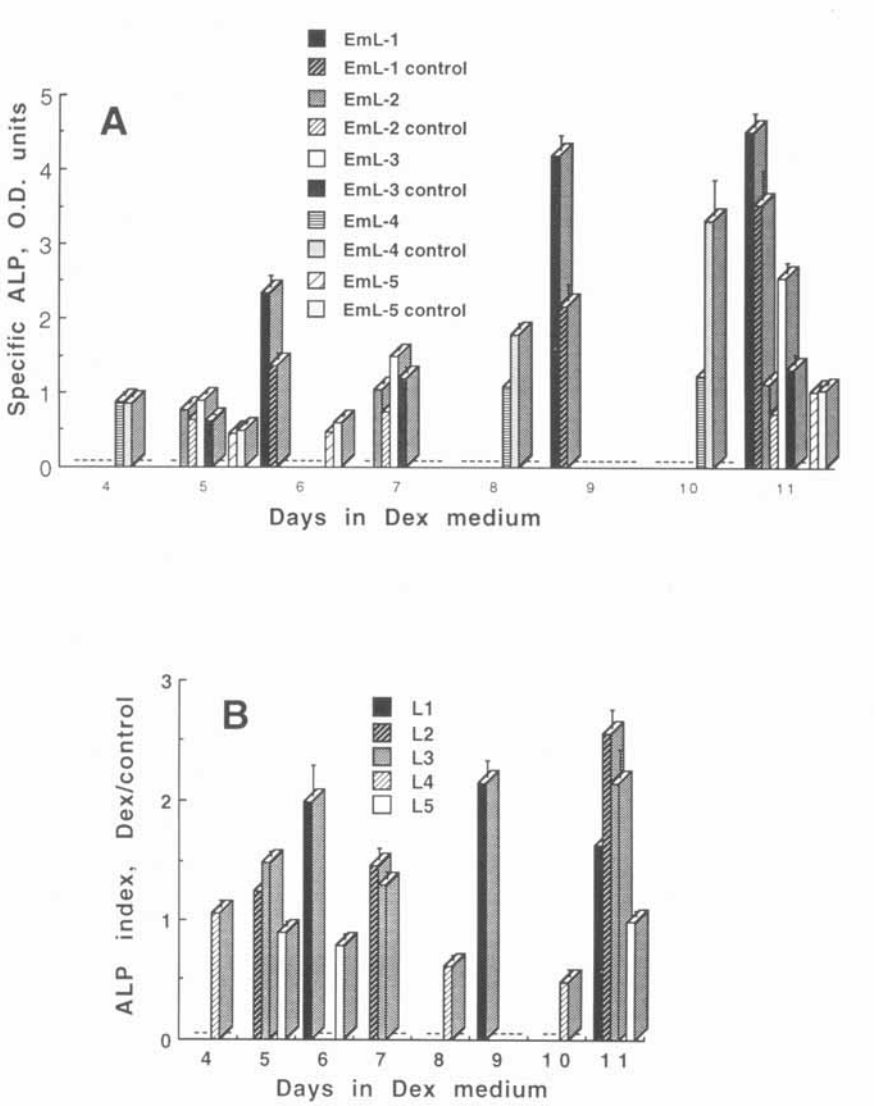

Fig. 2. Effect of Dex medium on specific ALP activity in embryonic bone cells of different individual origins. All were cells from the second culture transfer. Each cell line is represented three times between days 4 and 11. **A:** Dex-stimulated activities always on the left with their controls immediately following attached to their right. **B:** Dex/control ratios in which the index level of 1 represents the 0 change level (n = 20).

activity presented in Figure 2 regarding EmL-1(NF1), EmL-3(normal), and EmL-4(normal). Conversely, in EmL-2(HCv), cell proliferation was directly related to ALP activity, since the cell counts increased under Dex stimulation and, EmL-5(GD) cell proliferation also increased under Dex stimulation.

Cell-Mediated Mineralization in Culture

Figure 4 shows mineralization at day 21 of cell cultures (second transfer) under two different stimulation protocols. In Figure 4A, the Dex medium contained dexamethasone, ascorbate, and β-glycerophosphate (βGP), and the control medium consisted of ordinary medium supplemented only with ascorbate. The three cell cultures showed a similar mineralization response to Dex medium (close to 20 μg calcium/well), but their controls differed largely. EmL-1(NF1) cells showed 234 times less calcium/well in the control medium than the Dex-stimulated cells, whereas both "normal" EmL-3 and EmL-4 showed only twofold and fourfold less calcium, respectively. Under the second protocol (*Fig.* 4B), βGP was absent, up to day 11, from both the Dex-stimulated and controls and was added to both on day 11 for 10 days. In EmL-3(normal), dexamethasone decreased mineralization

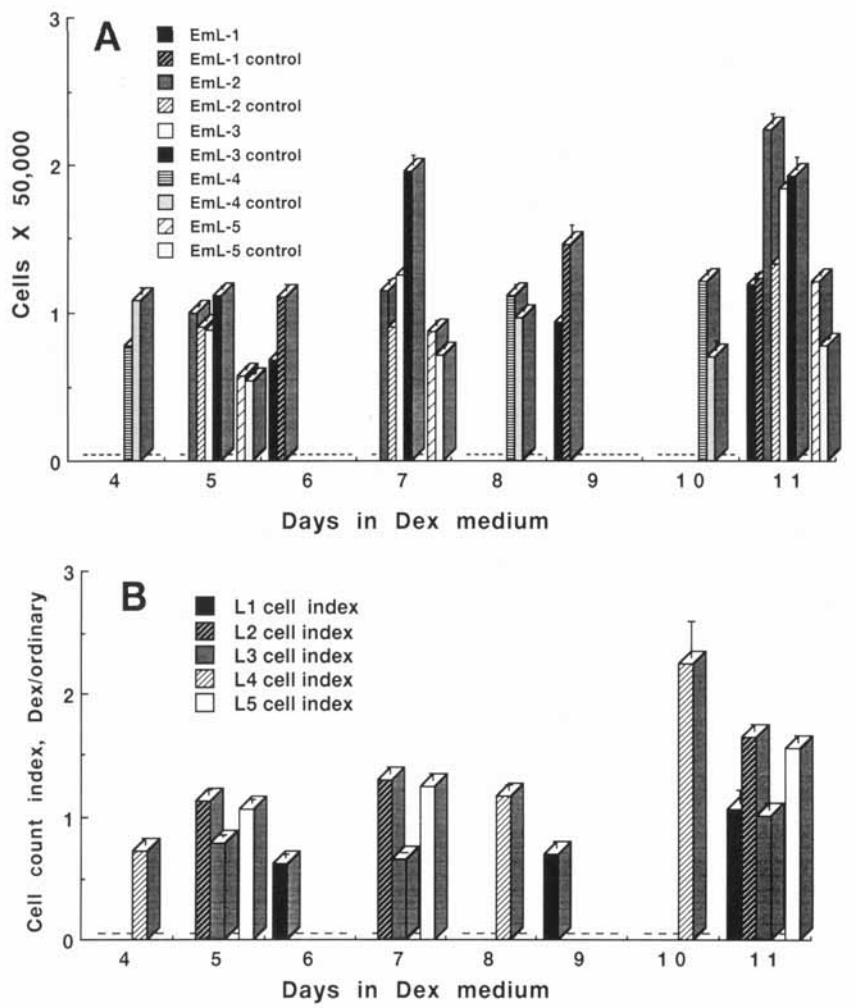

Fig. 3. Embryonic bone cells proliferation in respons to Dex medium. Results are presented at the same order as in Fig. 2.

slightly and in EmL-4(normal) and EmL-5(GD), it moderately decreased it, relative to the βGP-containing controls. In contrast in the EmL-1(NF1) controls, the mineralization was lower relative to the Dex-stimulated cells. A threefold calcium precipitation was observed in the Dex-stimulated cultures when βGP was present during the entire 21-day period (cf. Fig. 4A with 4B). EmL-1(NF1) clearly differs from other cells by its positive mineralization response to dexamethasone and also by its lower response to βGP when dexamethasone is absent.

ALP Activity and Cell Proliferation Under Malonate Selection

The cells that responded to dexamethasone by changing both osteogenic markers, ALP activity and mineralization, were tested for relative bone cell enrichment, using these markers, under malonate selection. Malonate inhibits succinate dehydrogenase conferring a selective advantage to cells undergoing an anaerobic stage. Day 3 of DEX stimulation was shown to be the most effective time for starting osteoprogenitor cell selection in rat stromal cell cultures [Klein et al., 1993a]. Therefore, day 3 was chosen for selection studies with 10 mM malonate in embryonic bone-derived cell cultures. Figure 5 shows day 11-specific ALP activity and cell proliferation responses of DEX-stimulated cultures of bone cells (4th culture transfers). At this cell passage, the absolute specific ALP activities of EmL-1(NF1) and EmL-3(normal) were comparable, whereas that of EmL-2(HCV) was 10-fold

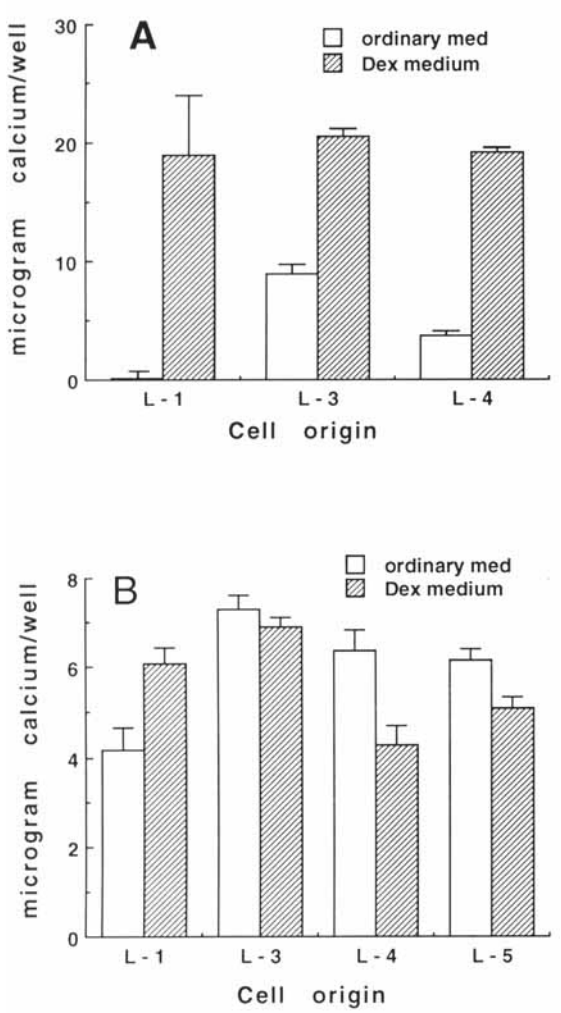

Fig. 4. Effects of βGP and βGP + dexamethasone on embryonic bone derived cell mineralization. **A:** Control cells were cultured from day 0 to day 21 with ordinary medium containing 50 μg/ml ascorbate and experimental cells in Dex medium (addition of dexamethasone + βGP). **B:** both ordinary and Dex did not contain βGP until day 11, when it was added to both media and maintained until day 21. The insoluble calcium precipitates were measured by the Arsenazo III binding method (n = 40).

lower and EmL-4(normal) was threefold lower (Fig. 5A). The ALP index of these cultures (Fig. 5C) was close to unity for EmL-1(NF1), "normal" EmL-3 and EmL-4, with a slightly lower cell count index, indicating that selection was futile or ineffective, since after day 3 of DEX stimulation, most of the cells were already malonate resistant. The EmL-2(HCV) cultures showed a greater than 40% increase in ALP activity index with out decrease in the cell count index. This indicates that in absolute terms, malonate induced a slight improvement in spe-

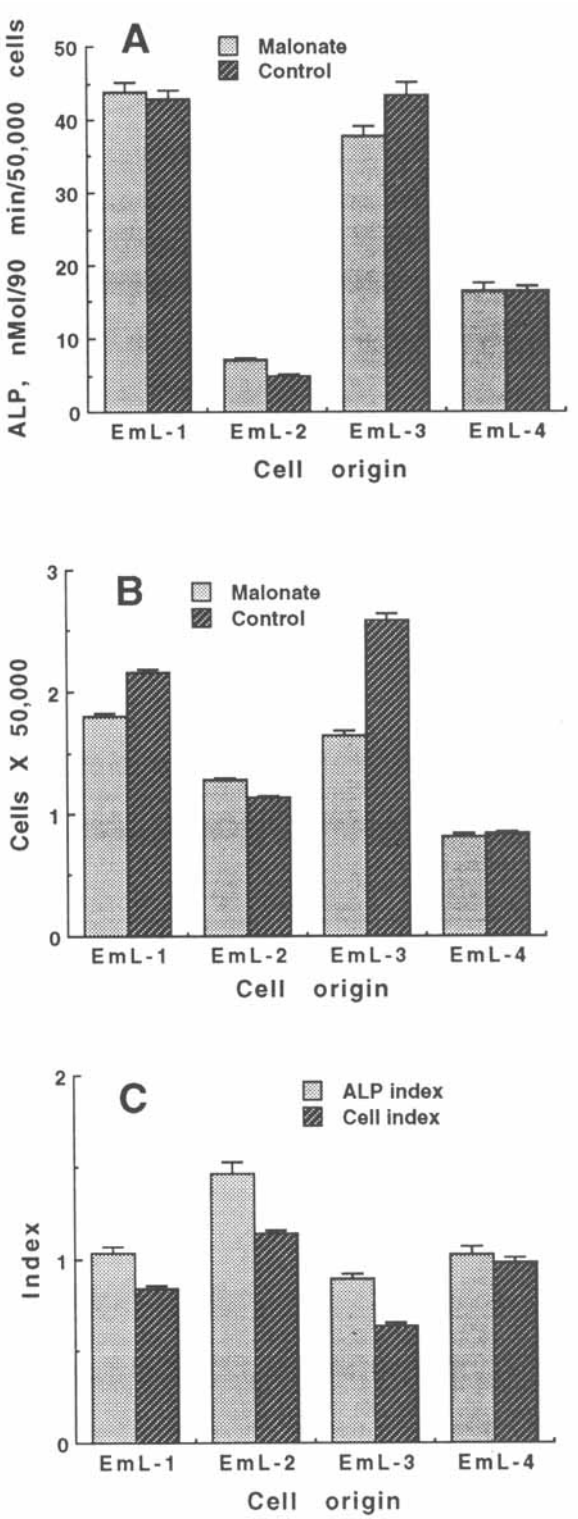

Fig. 5. Effects of malonate on specific ALP and cell count of Dex-stimulated embryonic bone-derived cells. All cultures were stimulated with Dex medium from day 0, the experimental half of the wells in each cultures was exposed to 10 mM sodium malonate from days 3 to 11. ALP (**A**) and cell counts (**B**) were performed on day 11 (n = 20). Index 1 (in **C**) designates 0 change.

cific ALP activity in EmL-2(HCV) cells (Fig. 5A). Malonate has slightly diminished the cell count in EmL-1(NF1) and EmL-3(normal) and slightly increased it in EmL-2(HCV); the cell count did not change in EmL-4(normal). In this fourth cell culture transfer, the DEX-stimulated cell counts of EmL-1(NF1) and EmL-3(normal) on day 11 were twofold higher than on the second cell culture transfer (Fig. 3B), malonate has decreased their cell counts, but more in the "normal" EmL-3 than in the EmL-1(NF1) cells. The DEX-stimulated absolute cell count of EmL-2(HCV) was rather lower than in the second transfer, and malonate treatment has slightly increased it, but far from its level seen in the second passage.

ALP Activity and Cell Proliferation Under Levamisole Influence

Levamisole was previously used to inhibit the early commitment to mineralization, by an unknown mechanism, rather than inhibiting the late (day 12–21) ALP activity. We showed that under such conditions it had the highest inhibitory effect on day 21 mineralization [Klein et al., 1993b]. Therefore, levamisole was also applied to embryonic cell cultures, of the dexamethasone-responding cells, from day 1 to 11. Figure 6A,C show that 0.2 mM levamisole induced a 40% increase of specific ALP activity in the EmL-1(NF1) without significantly affecting the ALP of normal EmL-3(normal) cells. Levamisole has also increased ALP activity in EmL2(HCV) but, contrary to the decreased proliferation in the former cells, its effect was accompanied by a slight increase in cell proliferation (Fig. 6B,C), EmL-4(normal) showed (Fig. 6C) an intermediated response between EmL-1(NF1) and EmL-3(normal).

Mineralization in Response to Levamisole

Figure 7 shows the day 21 mineralization by the dexamethasone-responding cells cultured with either levamisole or malonate during the first 12 days of Dex stimulation. Without levamisole, the absolute cell-mediated mineralization exhibited by the EmL-1(NF1) cells was at least twofold that of EmL-3(normal), EmL-4(normal) and EmL-2(HCV) (Fig. 7A). However, relative to their controls EmL-1(NF1), EmL-2(HCV) and EmL-4(normal) were much more resistant to levamisole inhibition than EmL-3(normal) (Fig. 7C). The absolute specific ALP activity of the

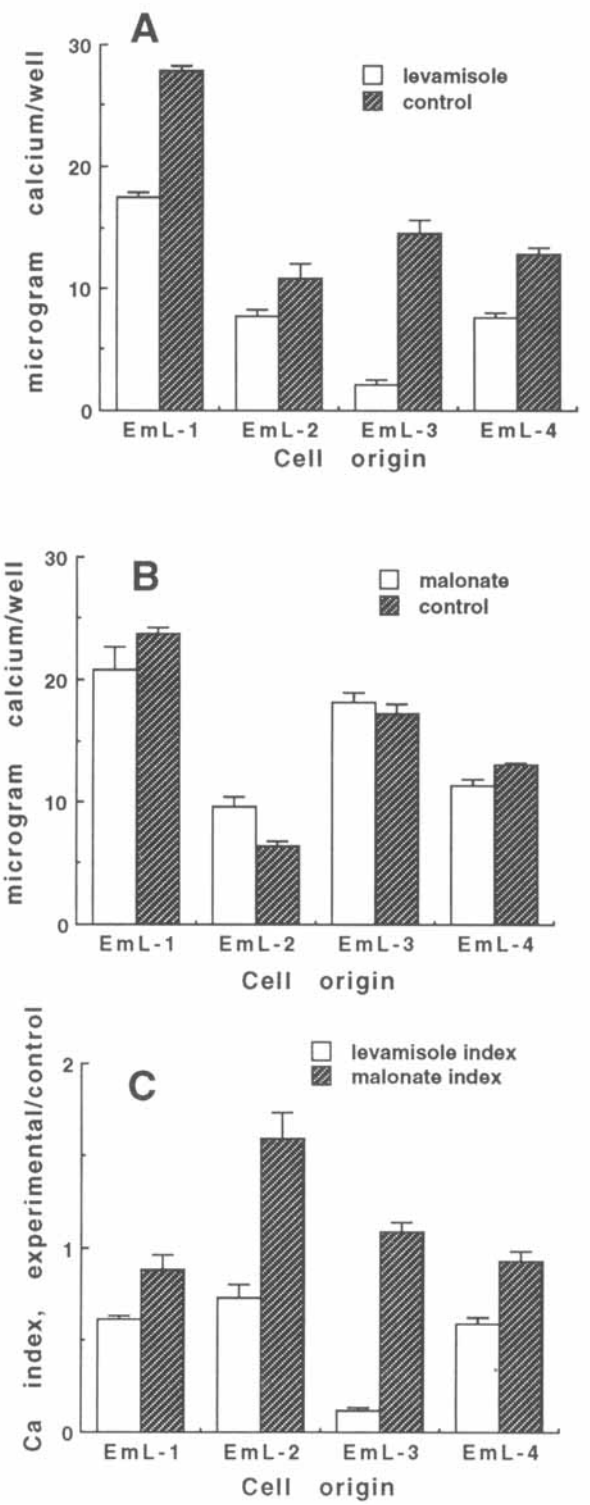

Fig. 6. Effects of levamisole on specific ALP and cell count of Dex-stimulated embryonic bone-derived cells. All the cultures were exposed to Dex medium from days 0 to 11, one-half of the wells were also exposed to 0.2 mM levamisole. ALP (**A**) activity and cell counts (**B**) were performed on day 11, (n = 20). Index 1 is the level of 0 change (**C**).

DEX-stimulated controls was directly related to the mineralization of DEX-stimulated controls in three cultures (cf. Figs. 6A and 7A). The "normal" EmL-4, the negative responder to Dex stimulation regarding ALP (Fig. 3), showed reciprocal mineralization and ALP responses to levamisole. Thus the effect of levamisole on mineralization was uncoupled from its effect on specific ALP activity, a phenomenon we also described in Dex-stimulated marrow stromal cells [Klein et al., 1993b].

Mineralization in Response to Malonate

The specific ALP activity with and without malonate (Fig. 5A) was directly related to mineralization (Fig. 7B) in all four cultures. The mineralization index under malonate (Fig. 7B), and the ALP index (Fig. 5C), were close to unity in both EmL-1(NF1), EmL-3(normal) and EmL-4(normal), and about 1.5 in EmL-2(HCV). This is in accord with the selection effect of malonate for mineralizing cells. The results indicate that malonate has exhausted its selection capacity for EmL-1(NF1), "normal" EmL-3 and EmL-4. However, in EmL-2(HCV), malonate increased mineralization by about 50% above the increase in the cell count, indicating that it has stimulated mineralization in EmL-2(HCV), rather than improving selection of mineralizing cells.

ALP Activity and Cell Proliferation in Response to cis-Hydroxyproline

This proline analogue, which inhibits hydroxylation of prolines during procollagen synthesis, was shown to increase ALP activity and mineralization in rat marrow stromal cells [Klein et al., 1994]. Figure 8 illustrates day 11 ALP responses of cell cultures to 5 mM cis-hydroxyproline. The culture responses to hydroxyproline were determined in two separate sets, in ordinary medium (Fig. 8A) and in DEX medium (Fig. 8B). The absolute specific ALP activity in DEX-stimulated cells was higher than in the respective cells grown in ordinary medium, as expected. The trans-isomer of hydroxyproline did not diminish ALP responses in cultures grown in ordinary medium, whereas cis-hydroxyproline substantialy diminished the ALP activity, more in EmL-1(NF1) than in EmL-2(HCV) and EmL-3(normal) (see differences in ALP index between left and right, Fig. 9A). Figure 9A (left) demonstrates the differential contribution of Dex medium to the cis-hydroxyproline effect.

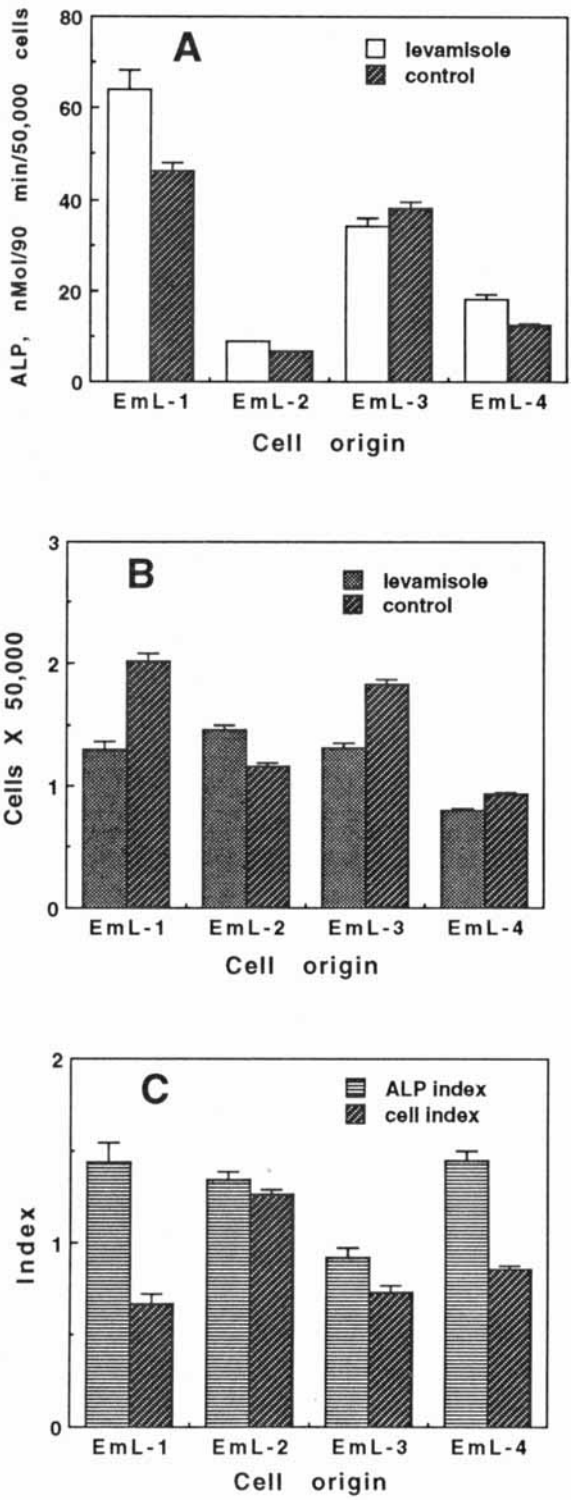

Fig. 7. Effects of early applied levamisole or malonate on cell-mediated mineralization by emryonic bone-derived cells. Dex-stimulated cultures were exposed on days 1–11 to 0.2 mM levamisole (A) or to 10 mM sodium malonate on days 3–11 (B) and were removed; on day 21, calcium precipites were measured (for each sample n = 40).

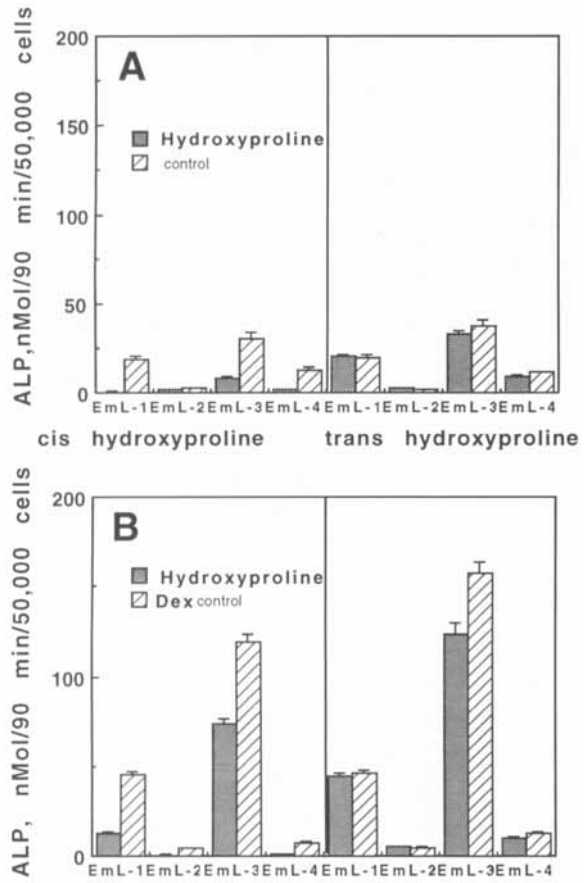

Fig. 8. Effects of cis-hydroxyproline on specific ALP activity in embryonic bone-derived cells, with and without Dex stimulation; cis- and trans-hydroxyproline isomers, 5 mM, were added on day 3 to experimental cultures which were either on ascorbate alone **(A)** or Dex-stimulated **(B)**. ALP activity was tested on day 11 (n = 10).

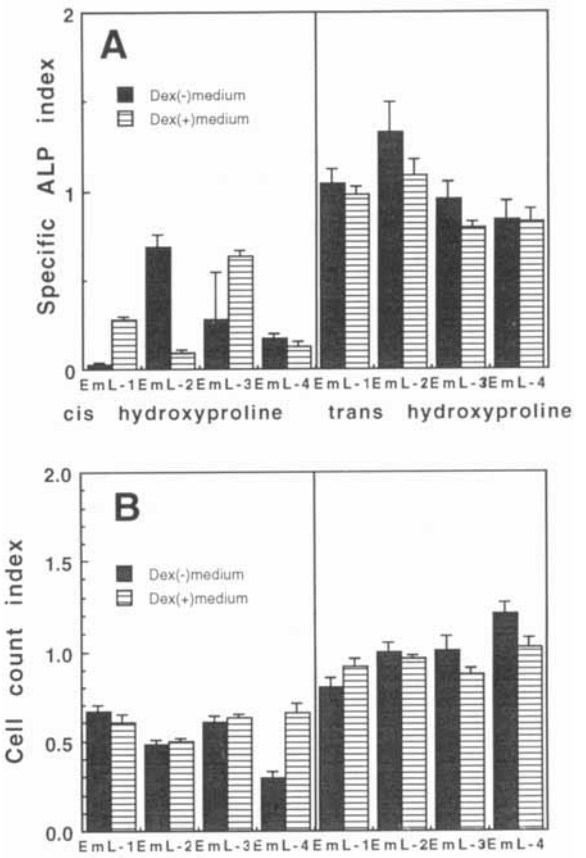

Fig. 9. Indices of specific ALP activity and cell counts induced by hydroxyproline isomers with and without Dex stimulation. Presentation of ALP indices **(A)** from Fig. 8 and cell count indices **(B)**.

EmL-1(NF1) is diagonally opposed to EmL-2(HCV) in its differential response to cis-hydroxyproline, and both differ from EmL-3(normal) and EmL-4(normal). The EmL-3 and EmL-4 cultures of "normal" origin do not show a significant differential response to cis hydroxyproline, regarding ALP index. It should be noted that, in EmL-3, the $Dex^{(-)}$ ALP index is not significantly different from that of the $Dex^{(+)}$, although the mean index is smaller. The ALP index change under cis-hydroxyproline was not accompanied by changes in cell count, neither direct nor inverse (compare left panels of Fig. 9A and 9B). In all cultures, unrelated to Dex stimulation, cis-hydroxyproline decreased cell proliferation (Fig. 10).

Mineralization Response to cis-Hydroxyproline

Mineralization by cells cultured in Dex medium decreased in response to cis-hydroxyproline (Fig. 11A) but did not change by trans-hydroxyproline except EmL4(normal) (Fig. 11B). The mineralization pattern in response to cis-hydroxyproline for cultures 1–3 (cis index in Fig. 11C) is similar to that of ALP and cell counts. This is shown by comparing with ALP indices of $DEX^{(+)}$ (Fig. 9A, left) and with cell indices (Fig. 10B, left). These results indicate that cis-hydroxyproline is either cytotoxic or inhibitory to part of the cells committed to mineralize.

DISCUSSION

Human bone-derived cells supposedly do not retain osteogenic potential in culture, even if grown in vivo in diffusion chambers [Ashton et

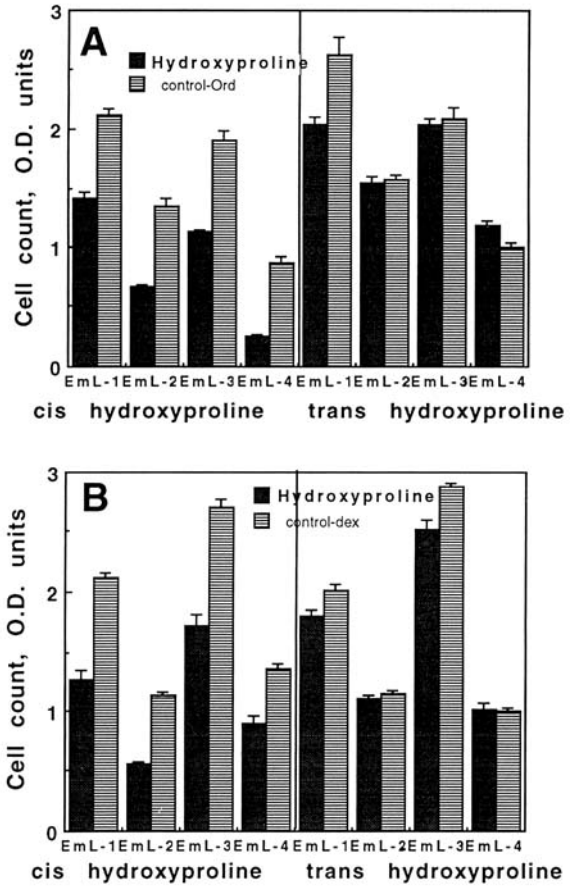

Fig. 10. Effects of cis-hydroxyproline on cell proliferation in embryonic bone-derived cells, with and without Dex stimulation. Absolute cell counts of cultures without Dex stimulation (**A**) and with Dex stimulation (**B**); 1.0 O.D. unit = 50,000 cells.

al., 1985]. However, the retention of osteogenic properties probably depends on the bone age, as Bab et al. [1988] showed that human bone marrow cells derived from a young donor did retain osteogenic capacity. This was also shown by Friedenstein [1976] to be valid for animal marrow stroma for which the number of osteogenic clones are inversely related to the donors age. In addition, retaining of osteogenic properties may also depend on the presence of ascorbic acid, which in the case of diffusion chamber culture is supplied by the host animal to the human cells that do not synthesize ascorbate de novo. Thus, supplying sufficient or long-acting ascorbate [Beresford et al., 1993] to human bone cells in culture may give rise to bone nodules, even in cells derived from adult bone. If indeed young age is advantageous for bone cells in retaining their osteogenic potential in culture, embryonic bone cells should be more capable of showing

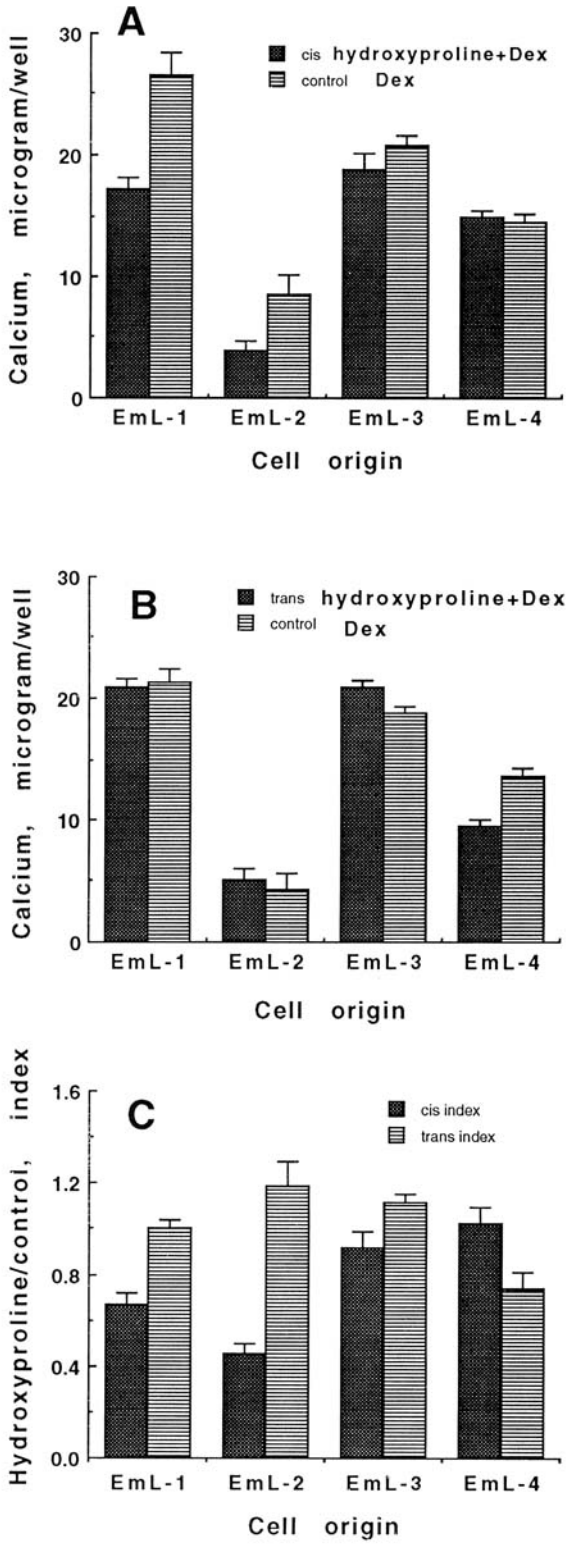

Fig. 11. Effect of cis-hydroxyproline on mineralization by Dex-stimulated embryonic bone-derived cells. Cultures have been treated as the Dex-stimulated ones in Fig. 8; on day 11, hydroxyproline isomers, cis (**A**) and trans (**B**), were removed. On day 21, calcium nonsoluble precipitates were measured, n = 40.

osteogenic characteristics in culture. The behavior in the culture of human embryonic bone-derived cells has not been characterized; recently, the first description of isolated human embryonic osteoblasts in culture has been published [Oliva et al., 1992]. We studied NF1 embryonic bone cells in parallel with cells of several other age matched controls, three without known genetic disorders, two of which were considered genetically normal.

The manifestations of NF1 (von Recklinghausen neurofibromatosis) are associated mainly with neuroectodermal tissues; however, this disease is also characterized by orthopedic manifestations. In many of these patients, the tibial arch is distorted; some have tibial pseudoarthrosis, which implies either a disturbance in mineralization or in an extracellular matrix component, or both. The bone-derived cell lineage in which a putative functional defect may exist is unknown; therefore, in the present study, bulk bone-derived cells were tested in culture. Since NF1 is a genetic disease, some of its phenotypic expression in embryonic cells was expected to be disclosed as a distinct alteration in one of the two bone differentiation markers: ALP and/or mineral. The high ALP activity in the unstimulated EmL-1 (NF1) cells was an outstanding feature relative to other cell cultures, it started early and also responded to Dex medium by an additional increase in its activity. There is no knowledge that connects high ALP activity with the NF1 phenotype. In human osteosarcoma tumor cells, such as Saos2, ALP activity is fivefold higher than in normal bone cells [Rodan et al., 1987]. Thus, based on the assumption that NF1 is a tumor suppressor gene, ALP activity should be expectedly high if connected to cell proliferation. This could be explained by neurofibromin (the NF1 gene product) deficiency, which may decreases Ras activity and increase the ALP gene expression via the AP1 transcription factor coplex.

The ALP response of "normal" EmL-4 to Dex medium was surprising; on day 10, unstimulated ALP activity was close to that of EmL-1 (NF1). However, contrary to the EmL-1 response, ALP substantially decreased under Dex stimulation in "normal" EmL-4. Since dexamethasone stimulates ALP expression, its paradoxical decrease in EmL-4 might be due to the presence of cells such as ALP-expressing preadipocytes and adipocytes [Gregoriadis et al., 1988; Beresford, 1989], which under continuous Dex stimulation show a decreased ALP activity [Dorheim et al., 1993]. We could not confirm morphologically the presence of adipocytes in the cultures. It should be noted that except in EmL-5(GD), ALP responses to Dex medium were reciprocal to cell proliferation responses, in line with the observation that expression of differentiation-specific markers is inversely related to cell proliferation [Stein et al., 1990].

The NF1 cells (EmL-1) were also outstanding in their inability to mineralize in the absence of βGP and dexamethasone (and with ascorbate) in contrast to "normal" EmL-3 and EmL-4. Dexamethasone clearly has a positive effect on EmL-1 mineralization; this effect was additive to that of the βGP. When βGP was added only on day 11 of Dex stimulation, dexamethasone had increased mineralization in EmL-1(NF1) above the effect of βGP, it had no effect on EmL-3(normal), and it has decreased mineralization in EmL-4(normal) and EmL-5(GD). These results indicate that some human embryonic bone-derived cells from 12 weeks gestation retain their osteogenic potential in culture, whereas neurofibromatosis bone cells may lose (or lack) this ability. This defect can be corrected by dexamethasone + βGP. The presented results are inadequate to suggest the mechanism by which dexamethasone affects mineralization by EmL-1(NF1) cells, namely, whether it is a simple effect directly on expression of several genes, or whether cell differentiation is involved. Alizarin-Red staining of mineralized nodules in day 21 EmL-1(NF1) cultures revealed mostly osteocyte-like dendritic cells in Dex-stimulated cultures, compared with mostly round cells in unstimulated controls (not shown). This morphological difference were not striking in the EmL-3(normal) cultures; i.e., in both cells, morphology has changes in response to Dex medium, but only in EmL-1(NF1) dexamethasone does it increase mineralization, suggesting that Dex medium had a combined differentiation and metabolic effect.

Malonate treatment of Dex-stimulated cultures did not result in a practical enrichment of mineralizing cells as in rat marrow stromal cells [Klein et al., 1993a], possibly due to a lower respiratory level in embryonic cells, making them more resistant to blocking of complex II enzymatic activity. This procedure did not distinguish the NF1 EmL-1 bone cell phenotype from other normal or diseased phenotypes tested under Dex stimulation. EmL-2(HCV) showed in-

creased mineralization in response to malonate, probably unrelated to the selection of mineralizing cells. EmL-2 cells originated from a woman infected with the hepatitis C virus and due to their atypical osteogenic characteristics in culture should be considered abnormal.

Exposure of the cultures to levamisole for the first 11 days has decreased the commitment to day 21 mineralization in all tested cells similarly to the effect shown in rat stromal cells [Klein et al., 1993b]. This effect, as in rat stroma, was uncoupled from ALP activity, which was increased in three of the four cells tested, probably resulting from the ability of levamisole to increase the expression of some membrane proteins [Kimball, 1993]. The application of levamisole, as that of malonate, was also not helpful in distinguishing the NF1 osteogenic phenotype from the phenotypes of the other cells.

The effect of cis-hydroxyproline, in Dex-stimulated cells, on the ALP activity relative to the controls enabled to discern EmL-1 from the other tested cells, especially when it was confronted with the effect on cells not stimulated with dexamethasone. The rational for using cis-hydroxyproline was its ability to block prolylhydroxylase [Kao and Prockop, 1977] during collagen synthesis, which occurs during cell proliferation. We have shown that this blocker has increased ALP and mineralization in differentiating rat stromal cells; in parallel, it increased mitochondrial respiration [Klein et al., 1994]. This effect differed from that shown in the present study, on the osteogenic parameters of embryonic bone cells. The interference of cis hydroxyproline with mineralization did not distinguish the EmL-1(NF1) phenotype from that of other cells as it did by interfering with ALP expression. It is thus possible that cis-hydroxyproline inhibits ALP expression via the pathway through which dexamethasone stimulates ALP, perhaps through the above-mentioned *ras* signal transduction pathway.

We conclude that osteogenic cells from 12th-week gestation embryos with NF1 should be useful in studying the regulation of cell-mediated mineralization in culture.

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