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Induction of Osteoprogenitor Cell Differentiation in Rat Marrow Stroma Increases Mitochondrial Retention of Rhodamine 123 in Stromal Cells

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Abstract Bone marrow stromal cells contain colony forming cells with the potential to differentiate into osteoprogenitor (OPC) cells. OPC-stimulation medium, containing dexamethasone, ascorbate, and β-glycerophosphate is widely used to recruit OPCs in culture. Cultures were incubated 24 h with rhodamine 123 (Rho), on different days, to examine the effect of the OPC-stimulation medium on the mitochondrial membrane potential of stromal cells. Cultures grown in both ordinary medium (DMEM with 15% FCS) and OPC-stimulation medium showed 2 Rho retention peaks on days 3–4 and 10–11. Between days 5 and 10 there was a drop in Rho retention/cell. OPC-stimulation medium increased Rho retention by at least twice the amount relative to ordinary medium, and has quadrupled it on day 7. Incubation with Rho concentrations above 5.0 μg/ml inhibited the portion of increased Rho retention which was contributed by the OPC-stimulation medium. Prolonged exposure to 0.1, 1.0, and 10.0 μg/ml Rho for 12 days only slightly increased day 12 ALP activity/cell, had no effect on day-21 mineralization and only the high dose, 10.0 μg/ml, doubled stromal cell proliferation. Under 24 h incubation Rho concentrations of 1.0 μg/ml and below can serve as a marker for mitochondrial membrane potential in differentiating stromal cells. The results indicate that under both culture conditions stromal cell mitochondria undergo cycles of high and low membrane potential states and that the OPC-stimulation medium constantly maintains an elevated membrane potential relative to ordinary medium.

Key words: energy metabolism, mineralization, OPC-stimulation, dexamethasone, mitochondrial membrane

Skeletal cells undergo a transient biochemical alteration during differentiation; their microenvironment becomes hypoxic [Brighton and Heppenstall, 1971] and their redox state is altered [Shapiro et al., 1982]. During local hypoxia growth plate chondrocytes rely on glycolysis as a source of energy under anaerobic conditions. The aerobic energy metabolism depends on reactions catalyzed by mitochondrial enzymes of the citric acid cycle. The mitochondria, in which the citric acid cycle occurs, also participate in controlling the cellular calcium metabolism in both soft and hard tissues.

The mitochondria in various soft tissues accumulate calcium [Carafoli and Sottocasa, 1984] by a metabolism dependent process, but mitochondria in skeletal cells presumably have an additional mechanism for calcium binding [Shapiro and Lee, 1975]. Calcium precipitation in the growth plate is preceded by calcium release from the mitochondria to the extracellular matrix [Brighton and Hunt, 1978]. Chondrocytic mitochondria accumulates calcium in a crystallized form [Lehninger, 1970] which is released when cartilage mineralization takes place. The mechanism whereby calcium is extruded from skeletal cell mitochondria has not been completely determined yet, but earlier studies provided some information about this process. It is known for long that cells under low oxygen tension restrict their energy utilization to the pre-mitochondrial glycolytic pathway [anaerobic metabolism, Krebs, 1953]. Such glycolytic metabolism was shown to exist between the proliferative zone and the hypertrophic condrocytes in front of the provisional calcification zone in the growth plate [Brighton et al., 1969]. Under anaerobic conditions molecular oxygen is less available in the

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mitochondria to support oxidative phosphorylation and to release NADH-derived protons. Correspondingly, under anaerobic conditions in hypertrophic chondrocytes of the growth plate, the NADH/NAD ratio is elevated [Shapiro, 1982]. This is consistent with lower cytosolic pH and accumulation of phosphoenol pyruvate, both characteristic of an anaerobic glycolytic metabolism, being associated with calcium release from chondrocytic mytochondria [Shapiro and Lee, 1978]. One of the results of oxidative phosphorylation supported by aerobic metabolism is to maintain a proton gradient across the inner mitochondrial membrane. Protons are expelled, electrons retained, intramitochondrial pH raises and negative charges increase in the mitochondria [Mitchell, 1966]. The negative charges have been exploited for illustration of the mitochondria by the fluorescent dye rhodamine 123 (Rho). This dye is hydrophobic, permitting easy crossing of cellular membranes. It is also cationic, which enables its binding to intramitochondrial negative charges. Rho can therefore serve as a probe for the proton gradient across the mitochondrial inner membrane [Chen, 1989].

Stroma derived osteoprogenitor cells (OPCs) presumably undergo a stage with characteristics of anaerobic metabolism during stroma cell differentiation [Klein et al., 1993a,b]. Marrow stroma derived OPCs undergoing proliferation can be recruited by dexamethasone [Bellows et al., 1987; Maniatopoulos et al., 1988; Leboy et al., 1991; Kamalia et al., 1992]. In the present study we tested the effect of dexamethasone containing OPC stimulation medium on Rho retention under the same conditions used to recruit stromal cell derived OPCs, while they differentiate into mineralizing bone nodule in culture.

MATERIALS AND METHODS

Reagents

ALP kit 104 LL, dexamethasone, ascorbate, β-glycerophosphate, and rhodamine 123 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. Stromal cells were obtained as described by Maniatopoulos et al. [1988], removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were propagated in the same maintenance medium, which consisted of DMEM (Dulbecco modified Eagle's medium) supplemented with 15% fetal calf serum and antibiotics, in a humid 10% CO_2 atmosphere. For the experimental cultures stromal cells were removed 2 weeks later by trypsinization and were plated in 96 well microtiter plates, 5000 cells/well, and grown in osteoprogenitor cell (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) stock solution ×20 in distilled water was added to the cultures, 10 μl/well, after an incubation period as indicated, mostly 24 h, the Rho-containing medium was removed. The cells were washed twice with TNC (50 mM Tris, pH 7.6, 150 mM NaCl) and continued their cultivation after the short incubation period until Rho retention measurement on the following day. For the 24 h or longer incubation periods cultures were prepared for Rho retention measurement immediately after removal of Rho.

After removal of the growth medium and washing with TNC the cells were incubated for 30 min at room temperature 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 assay cells at the lower half of the plates were stained using the methylene blue (MB) method [Goldman and Bar-Shavit, 1979]. 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 minutes. O.D. of the eluted MB was measured at 620 nm by a multichannel spectrophotometer, 1.0 unit is equivalent to 5×10^4 stromal cells.

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,b]. 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 hydrolysed pNPP was measured in a multichannel spectrophotometer at 405 nm wave length. ALP specific activity was expressed either as nMol/time/cell, or as an activity index (experimental/control specific ALP ratio).

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 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 and presented as indices of experimental to control ratios.

Fluorescent Microscopy

Stromal cells were seeded on sterile, round coverglasses which fit by diameter into 12-well tissue culture plates. Cells, 4×10^4 in 0.2 ml of ordinary medium, were carefully loaded on the coverglass center inside the well and left to settle for 4 h in the incubator. The medium was then adjusted to 2.0 ml/well. After 3 days (day 0) the medium was changed to ordinary and OPC stimulation medium for controls and experimental cultures, respectively. One day before the microscopic examination the medium in each well was adjusted to 1.0 μg/ml of rhodamine 123 (Rho) for a 3 h incubation. After removal of the medium, cells were washed twice with PBS and

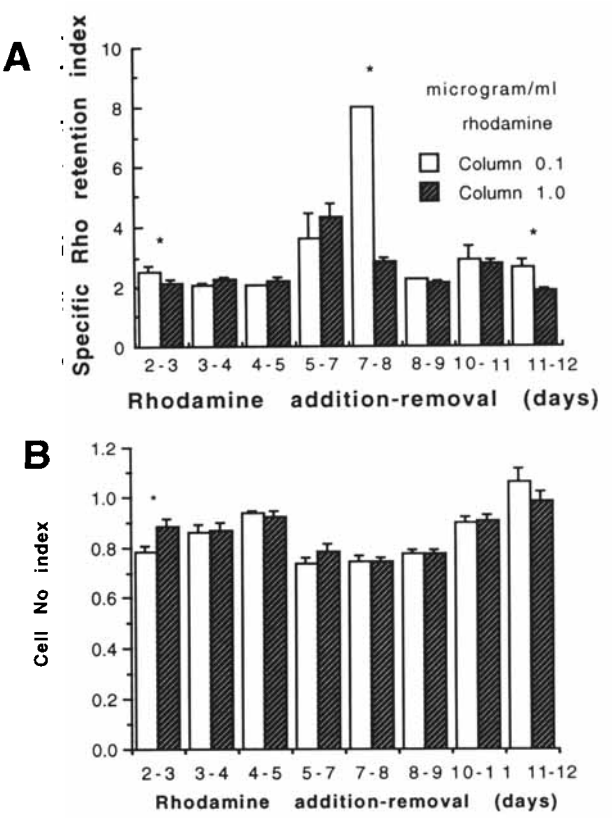

Fig. 1. Effect of OPC stimulation medium on stromal cell retention of Rho after 24 h presence in culture. Stromal cells cultured in OPC stimulation and ordinary medium in each of 8 microtiter plates were exposed to 2 Rho concentrations, each plate on a different day. After 24 h Rho was removed. Cells were then washed twice with PBS, lysed (**A**), and the lysate fluorescence measured by a spectrofluorometer. For each sample the mean ± SE of experimental to control ratio is presented as an index of Rho/cell, n = 10 pairs of experimental and control wells. The lower panel (**B**) represents in a similar fashion the cell count index. Cell counts were obtained from the opposite lower half of the plate. Astrix = significant difference between results of both Rho concentrations within each time point.

were incubated, each well with its appropriate medium, for additional 24 h. Subsequently, the next day, each coverglass was removed separately from its well and mounted, cell side down, on a glass slide and inspected under a Zeiss fluorescent microscope. Cells were photographed with an attached camera using an Olympus exposure control unit, through a Planapo ×40/oil immersion objective.

RESULTS

OPC Stimulation Medium Effect on Rho Retention at Different Days

Figure 1A shows that rat bone marrow stromal cells cultured in OPC stimulation medium and with 0.1 or 1.0 μg/ml Rho for 24 h retain

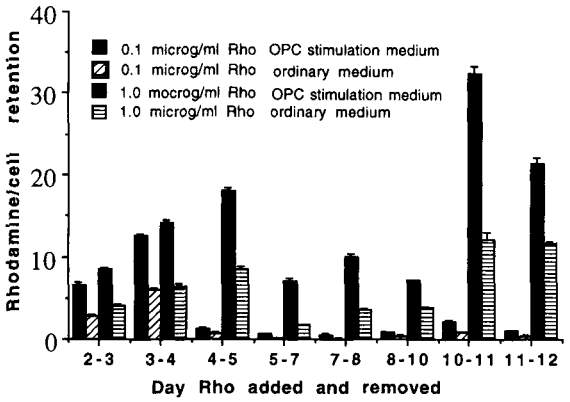

Fig. 2. Profiles of stromal cell Rho retention during 12 days in culture with ordinary and with OPC stimulation medium. Rho retention/cell mean ± SE are presented for two Rho concentrations: 0.1 and 1.0 μg/ml, n = 10. The incubation periods with Rho lasted 24 h except for the day 5–7 period of 48 h.

Rho at least twice as much as the controls grown in ordinary medium. On days 5–7 and 7–8 there was a further relative increase in Rho/cell retention, 4–8 times, respectively. On days 2–3, 7–8, and 11–12 cultures exposed to 0.1 μg/ml Rho have retained a greater proportion of the dye than those exposed to 1.0 μg. In parallel there was little difference in cell quantity under both Rho concentrations.

Figure 2 shows a comparison between absolute values of Rho retention in ordinary medium and OPC stimulation medium cultures. Peaks on days 3–4 and 10–11 can be seen under both media, Rho retention/cell dropped between days 5 and 10. The abrupt drop in Rho retention between measurements on day 5 and day 7 is consistent with the prolonged incubation with the dye, 48 h (days 5–7) compared with 24 h for all other incubation periods. Figure 2 illustrates the occurrence of an interim period of a drop in Rho retention which started after the day 3–4 period for the low (0.1 μg/ml) Rho concentration and after days 4–5 for the higher (1.0 μg/ml) concentration. This drop in Rho retention/cell was seen in stromal cells cultured with both ordinary and OPC stimulation medium. The OPC stimulation medium increased the specific Rho retention. According to Figure 1, day 3 and day 7 were suggestive to exemplify a weak and a strong Rho-dose dependence, respectively. Therefore day 3 and day 7 Rho retention were chosen for quantitative analysis of the Rho dose effect on its own retention.

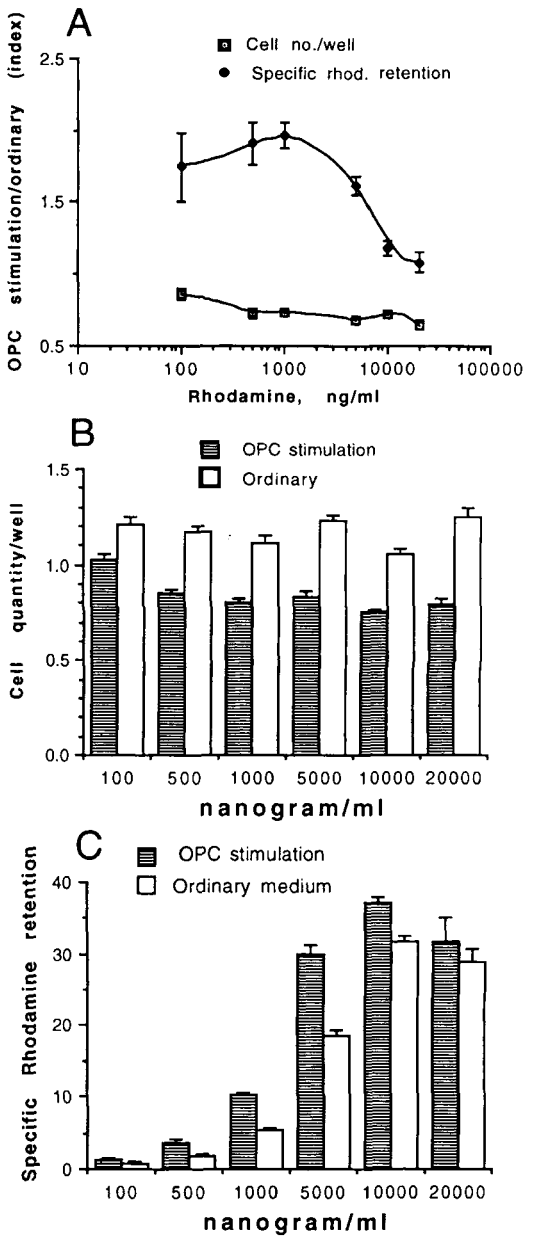

Fig. 3. Effect of Rho concentration on its own retention on days 2–3. Different doses of Rho were added to the cells on day 2 and handled as in Figure 1. **A:** Indices of cell count and Rho retention/cell. **B:** Absolute values of experimental and control cell counts. **C:** Absolute values of Rho retention. Dexamethasone = OPC stimulation medium, n = 10.

Rho Dose Response on Days 2–3 and 6–7

Figure 3 illustrates the effect of OPC stimulation medium on stromal cell retention of Rho under various Rho concentrations, on days 2–3 of stimulation. OPC induction only slightly decreased the cell count (on days 2–3) at all Rho concentrations (Fig. 3A,B). However, a dose dependent Rho retention/cell is shown in Figure

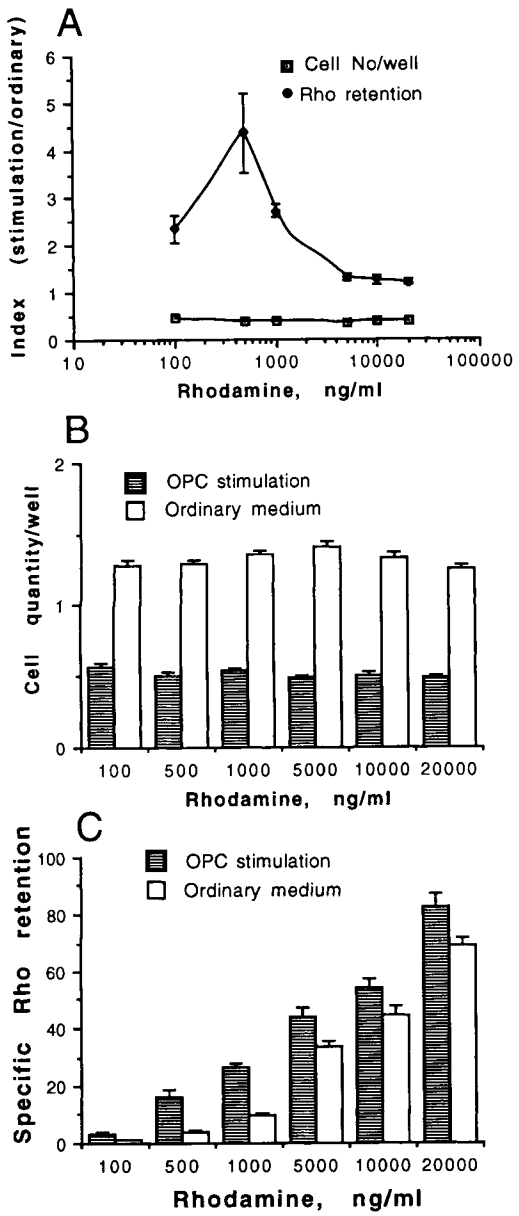

Fig. 4. Effect of Rho concentration on its own retention on days 6–7. Dose response of Rho effect on its retention measured on day 7 similar to the experiment described in Figure 3.

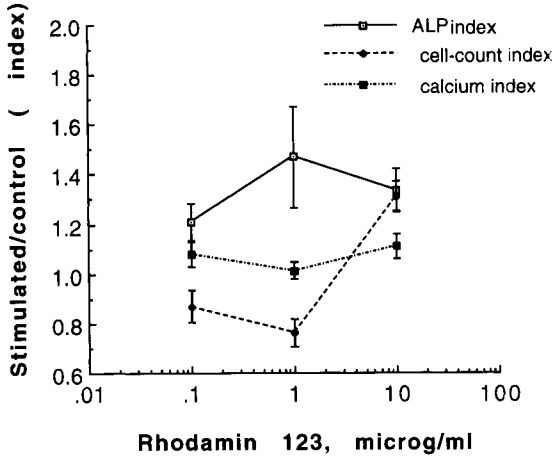

Fig. 5. Effect of Rho on stromal OPCs differentiation. Stromal cells cultured with OPC stimulation medium was at the presence or absence of 0.1, 1.0, and 10.0 μg/ml Rho, were assayed on day-11 for ALP/cell activity, cell count and on day 21 for cell mediated calcification. The medium was changed every 3–4 days, and up to day 11 the fresh medium contained appropriate Rho concentrations, n = 20.

3C for both stimulated and control stromal cell cultures. Unlike the cell count the specific (per cell) Rho retention was substantially higher in stimulated than in the control cultures (Fig. 3C). Therefore, the Rho retention index in Figure 3A is close to 2.0 (as in Fig. 1A for 0.1–1.0 μg/ml [Rho]), but it decreases, becoming closer to 1.0 at 20.0 μg/ml Rho (Fig. 3A).

Figure 4 shows the effect of OPC induction medium in stromal cells, in Rho retention on day 7, under similar Rho concentrations as in Figure 3. Here the absolute values for the dose response of Rho retention/cell were higher than on day 3 (Fig. 4C), the cell counts of stimulated cells are lower than in the controls, but relative to control cultures they were similar at all Rho concentrations (Fig. 4B). Thus upon 24 h incubation various Rho concentrations do not change the cell count ratios, but Rho concentrations between 0.1 and 1.0 μg/ml show a retention index between 2.0 and 5.0 (Fig. 1A) and from 5.0 μg/ml the index is decreased becoming closer to 1.0. Notice that Rho retention for 1.0 μg/ml are higher in Figure 4C than the respective values of days 5–7 in Figure 2. This is due to prolonged incubation of 48 h, which may decrease retention compared to 24 h incubation.

Effect of Continuous Culture With Rho on OPC Differentiation

Figure 5 shows that 0.1, 1.0, and 10.0 μg/ml Rho kept in OPC cultures for 12 days (renewing the Rho concentrations during medium changes) caused a slight increase in day 11 ALP activity (1.2–1.45 times higher than controls grown without Rho) and a slight decrease in cell counts (0.9–0.75) at 0.1 and 1.0 μg/ml but at 10.0 μg/ml of Rho cell counts slightly increased. Day 21 results of calcium/well caused by cell mediated mineralization was barely affected by these Rho concentrations.

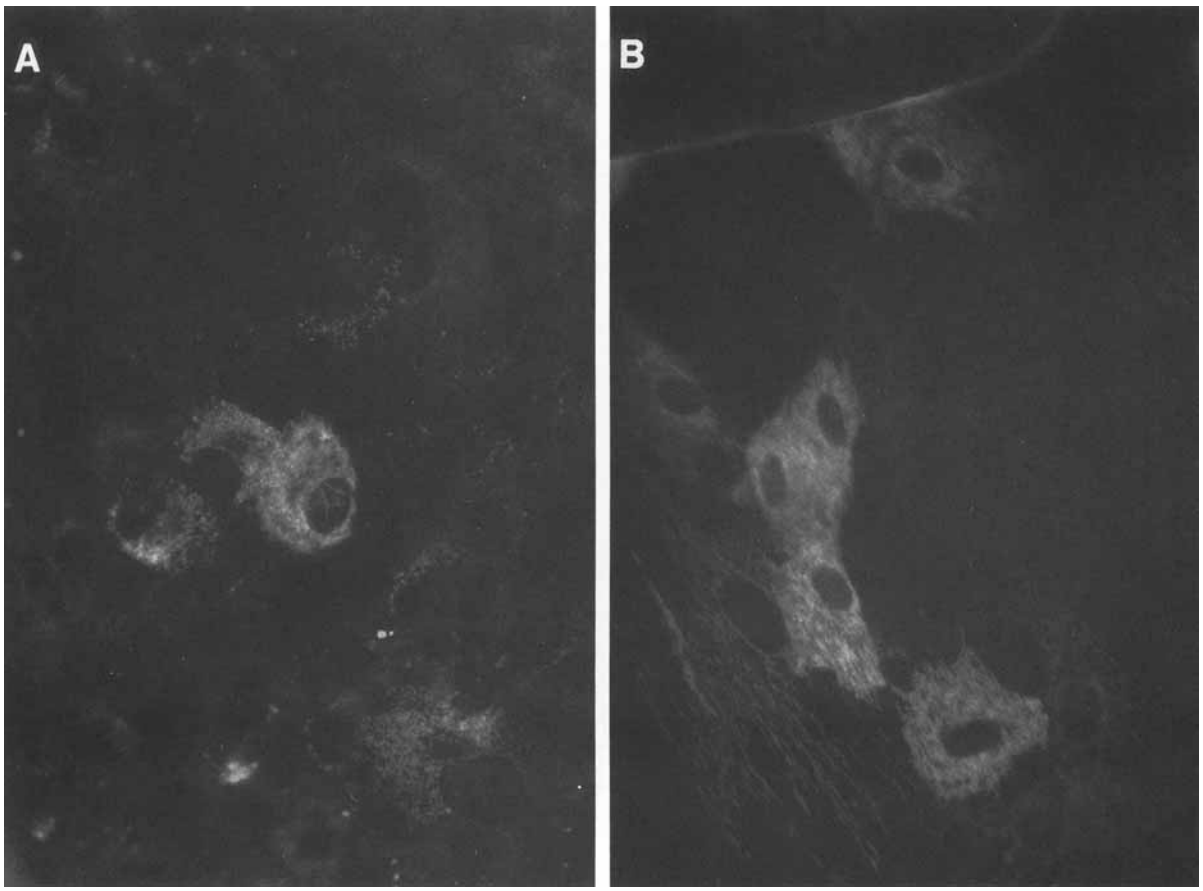

Fig. 6. Fluorescent microscopy of day 7 intracellular Rho retention. Stromal cells cultured with and without OPC stimulation medium on round coverglasses were exposed for 3 h to 1.0 μg/ml Rho and photographed 24 h later under a fluorescent microscope. **A:** Cultured in ordinary medium. **B:** Cultured in OPC stimulation medium (magnification ×400).

Microscopic Presentation of Intracellular Rho Retention

Days 6–7 of stroma cultured with OPC stimulation medium showed a substantial increase in Rho retention relative to controls; therefore, Rho retention was tested on day 7 by visualization of the retained Rho. Figure 6A shows a diffuse fluorescent image of stromal cells which were cultured in ordinary medium. The confluent and slightly smaller cells constitute a background to a cell with a fluorescence intensity similar to the positive Dx-stimulated cells seen in Figure 6B. Figure 6B shows a fluorescent image of stromal cells which were 7 days in culture with OPC stimulation medium. The cells are bigger than most of the non-stimulated cells seen in Figure 6A and the intracellular fluorescence is not diffuse, showing more distinct intracellular structures either round or prolonged organells consistent with the shape of mitochondria [Chen, 1989]. Figure 6B shows fewer cells than in Figure 6A, which is in accord with the lower cell count index shown in Figure 4A, being also consistent with the high (2.5) Rho retention index, seen at the same Figure, for 1.0 μg/ml Rho (1,000 ng).

DISCUSSION

In the present work changes in Rho retention by mitochondria of differentiating bone-marrow stromal cells-derived OPCs were studied. Dexamethasone (Dx), 0.1 nM, is able to recruit self-renewing OPCs in stromal cultures as measured by quantitative cell mediated mineralization [Kamalia et al., 1992]. Therefore it was interesting to follow the effect of Dx-containing OPC stimulation medium on mitochondrial membrane potential during OPCs differentiation. Rho was retained in stimulated cultures at least twice as much as in unstimulated cultures. During the period of the drop in Rho retention, days 5–8, Rho retention has quadrupled under Dx contain-

ing medium relative to ordinary medium cultures. The cell counts showed only small differences between stimulated and unstimulated cultures (Fig. 1B). Profiles of Rho retention/cell followed in stimulated and unstimulated cultures showed peaks on days 3–4 and 10–11 (Fig. 2). This suggests the existence, between days 4 and 10 from the start of stimulation, of a lower inner membrane potential because of a weaker "proton motive" activity. The reducing capacity of dexamethasone-stimulated stromal cell cultures, as measured by a tetrazolium salt (XTT), peaked on day 3 and declined from day 5 to day 10 (unpublished data), this corroborates the pattern of low concentration, 0.1/ml, Rho retention (Fig. 2). It is thus possible that the Rho retention peak on days 3–4 is the result of an adequately functioning Krebs cycle under aerobic conditions, from day 0 to 3, enabling an adequate NADH/NAD ratio to supply protons for efflux by the F_0F_1-ATPase (ATP-synthase). The abrupt drop in Rho retention is probably the result of decreased proton efflux, which may be due to several factors.

High concentrations of Rho (from 20 μg/ml) can be cytotoxic, especially for tumor cells [Lampidis et al., 1983]; the main target for the toxic effect is ATP synthase [Modica-Napoletano and Aprille, 1987], being one of the enzyme complexes on which Rho depends for its retention. Since Rho may inhibit its own retention, we paid attention to differences in its accumulation at different concentrations. Rho retention was enhanced using the 10-fold (1.0 μg/ml Rho) concentration relative to 0.1 μg/ml.

According to the dose curve of Rho retention on days 2–3 and 6–7 the dose range at which OPC stimulation medium was distinguishable from ordinary medium was between 0.1 to 1.0 μg/ml of Rho upon 24 h exposure to the dye. At 10.0 and 20.0 μg/ml, Rho has abrogated its own retention (Figs. 3, 4). Cell counts are slightly decreased with 0.5 μg/ml Rho on days 2–3, but were practically insensitive to all doses on days 6–7. Upon prolonged exposure to 0.1, 1.0, and 10.0 μg/ml Rho for 12 days of Dx stimulated cultures against controls of Dx-stimulated cultures but without Rho, the dye did not affect day 21 mineralization and had only slightly increased ALP activity/cell with no significant difference between the various doses. This indicates that the effect of Rho on ATP-synthase is not sufficient to change the commitment of OPCs to mineralization during differentiation. Twelve days exposure to 10.0 μg/ml relative to 1.0 μg/ml Rho had a proliferative effect on stromal cells being an interesting phenomenon per se. The data are not sufficient to determine whether high dose Rho and prolonged exposure cause proliferation only in OPCs or also in other stromal lineages. It can be concluded that doses below 1.0 μg/ml Rho do not interfer with OPCs differentiation upon 12 days exposure to the dye. Upon 24 h incubation doses below 0.1 μg/ml do not abrogate the Dx medium-induced increase in Rho retention according to an index of > 1.0 seen in Figures 3A and 4A. Therefore Rho at such low concentrations can serve as a marker for transient changes in the mitochondrial membrane potential of intact stromal cells differentiating into OPCs. The rational for the use of Rho to follow calcified tissue differentiation is the hypothesized linkage between the mitochondria as a calcium source, the importance of c-fos in osteogenic differentiation [De Togni et al., 1988], the dependence of Rho retention on v-fos expression in fibroblasts [Zarbl et al., 1987] and the existence of a fos transformation effector gene whose product may be involved in mitochondrial protein import [Kho and Zarbl, 1992].

Stromal cells cultured with OPC stimulation medium may contain adipocytes and reticular cells [Owen and Fridenstein 1988; Beresford, 1989] the distribution of Rho retention among them and the OPCs is not clear. This may be clarified by Rho retention studies in differentiating stromal cells under mineralization-inhibitors and enhancers which are now in progress. This may be accomplished due to the ability of some of these compounds to enrich OPCs in the stromal cell cultures [Klein et al., 1993a].

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