

## Expression of Alkaline Phosphatase during Osteogenic Differentiation of Rat Bone Marrow Stromal Cells

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### Abstract

**Introduction:** Bone marrow contains a population of stem cells capable of differentiating to osteoblast and forming the bone nodule by dexamethasone.

**Material and Methods:** The stromal cells of bone marrow obtained from 4 to 6 weeks old Spruge-Dawely male rats were grown in primary culture for 7 days and subcultured for 18 days. The cells were cultured in either DMEM medium containing 15% fetal calf serum and antibiotics as the controls or the above medium supplemented with osteogenic supplements (OS): include 10 mM Na-β glycerophosphate (Na-βGp), 10 nM dexamethasone (Dex) and 50 g/ml ascorbic acid (AsA) as the examined cultures. After 6, 12 and 18 days of grow up in subculture, the cultures were examined for mineralization and alkaline phosphatase (Apase) expression.

**Results:** Mesenchymal stem cells (MSCs) in examined cultures underwent a dramatic change in cellular morphology and a significant increase in Apase activity by day 12. The deposition of a calcified matrix on the surface of the culture flasks became evident between days 12 and 18.

**Conclusion:** The addition of osteogenic supplements (OS) to MSCs cultures induced Apase expression that contributes to cellular differentiation and mineralization of extracellular matrix.

**Key words:** Alkaline phosphatase, Bone marrow, Mesenchymal stem cells (MSCs), Osteogenesis, Culture, Rat

## Introduction

Osteoblasts are believed to be derived from multipotential stem cells in the bone marrow. These stem cells have both a capacity for self-renewal and under the influence of unknown factors, have been proposed to give rise to progenitors of each mesenchymal cell types: fibroblasts, myoblasts, adipocytes, chondroblasts, osteoblasts and reticular cells. For this reason, Haynesworth has termed these: marrow-driven mesenchymal stem cells. Evidence supporting their osteogenic and chondrogenic potential includes the ability of bone marrow suspensions to generate cartilage and bone when transplanted into diffusion chambers in vivo. Bone marrow stromal cells (BMSCs) have long been recognized as a source of osteoprogenitor cells which named as mesenchymal stem cells (MSCs). BMSCs are fibroblasts-like cells that are thought to include precursors for a number of different connective tissue cell phenotype, including osteoblast. Fridentein has termed this cells "determined osteogenic progenitor cells" (1). In vitro, stromal cells from rat, mouse, and human bone marrow have been shown to give rise to mineralizing cultures with the characteristics of osteoblasts. Potential regulators of osteogenic differentiation from marrow stromal cells include soluble and bone matrix-driven factors.

Numerous factors are known which exert modulatory effects on cells with the osteoblastic phenotype. The synthetic glucocorticoid, dexamethasone and the bone morphogenic proteins, are specially noteworthy because they induce expression of osteoblastic phenotypic markers in both immature osteoblasts and in less-committed cells. It has been demonstrated that BMSCs isolated from bone marrow can be induced by glucocorticoids to form the bone nodules. Furthermore Dex treatment of Apase-negative osteoprogenitor change them into Apase-positive cells possessing ability to form mineralized bone nodules (2, 3).

During differentiation in vitro, markers of osteoblast phenotype appear, such as accumulation of extracellular matrix, expression of alkaline phosphatase (Apase) and mineralization of bone nodules.

These findings indicate that this culture system may be a useful model for investigating the factors that stimulate the differentiations of osteoprogenitor cells and regulate the expression of the osteoblastic phenotype (4, 5).

Apase has been studied for more than 70 years (6). This enzyme belongs to a family of proteins that are anchored to the plasma membrane via a glycosylphosphatidinositol linkage (7, 8). Apase is widely used as a marker of the osteoblast phenotype, not least because of the simplicity with which it can be assessed. Osteoblasts express the tissue-non specific form of the enzyme. High levels of Apase are seen in both preosteoblasts and osteoblasts in vivo and in differentiating osteoblasts in vitro. The biological functions of Apase are still unknown except for a role in bone mineralization. Connective tissue cells expressing high levels of Apase can be induced to deposit mineral in vitro (9). In a human model of Apase deficiency, hypophosphatasia, the bone becomes soft in the absence of Apase activity, suggested that Apase physiological function is to maintain bone mineralization after birth (10). More recently two mouse Apase knockout models showed that, when the gene for Apase specifically deleted, the mice developed defects in bone mineralization shortly after birth but not necessarily during embryonic development. It is possible that unknown redundant or backup mechanisms provide for mineralization during embryonic life in Ap-deficient knockouts, meaning that Apase could still play an important role in the initial mineralization processes in bone (11, 12).

These results could suggest that Apase play an important role in the maintenance of bone mineralization.

## Material and Methods

### \* Cell cultures

Examined cultures were carried out in DMEM medium with 15% fetal calf serum, ascorbic acid (50 µg/ml), dexamethasone (10 nMol/L), Na-β glycerophosphate (10 nM) and antibiotics in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C (3, 13, 14). Control cultures were carried out in above medium

without any supplements.

Bone marrow cells were obtained from the femur of 4-6 week-old male Spruge-Dawely rats. Animals were killed by overdose chloroform. Both femora were removed and the soft tissues were detached aseptically, metaphyses from both ends were resected and bone marrow cells were collected by flushing the diaphysis with a culture medium. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 20 gauge needle, cells obtained from each femur, plated in 250 ml tissue culture flasks separately and cultured for 7 days by replacing the medium every 2 days. On day 7, subcultures were prepared.

Cells were washed with warm phosphate-buffered solution (PBS) and adherent cells were detached by using Trypsin-EDTA. Trypsinized cells were passed through a syring with a 20 gauge needle to make single-cell suspension, counted with a hemacytometer and plated in 50 ml tissue culture flasks at a density of  $4 \times 10^3$  cells/cm and cultured for 20 days by replacing the medium every 2 days.

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#### \* Culture examination

Cultures were examined daily using phase contrast microscopy (13), for examining the morphology of cells. Some cultures were fixed in 10% neutral buffered formalin and stained with either H&E light green.

#### \* Mineralization assay

Selected specimens after 6, 12 and 18 days of grow up in subculture were satined for mineral deposition by Von Kossa method (15). Cell layers were fixed with 10% neutral buffered formalin for 1h, incubated with 2% silver nitrate solution for 10 min in dark, washed throughtly with deionized water and exposed to bright light for 15 min. The stained cultures were examined using phase contrast optics.

#### \* Alkaline phosphatase assay

Selected cultures were fixed in cold (4°C), 10% neutral buffered formaline after 6, 12 and 18 days of grow up, and incubated for 10 min with sodium-

$\alpha$ -naphthyl phophate in "tris" buffer, pH 10 in the presence of Fast Red Violet LB salt. During incubation, the cultures were protected from drying and direct light. The stained cultures were rinsed with dionized water, and air-dried, then were examined using phase contrast optics (16). Statistical signification was determined using student t-test.

## Results

### \* Cell morphology

MSCs cultured with OS (containing 100 nM Dex, 10 nM Na- $\beta$ -GP and 50  $\mu$ g/ml AsA) showed a change in their morphology from spindle-shape to cuboid in as little as 4 days and was more apparent by days (Fig. 1a) in the from of stormal cell colony.

While control cultures grew as uniform sheet of cell, by day 12 (Fig. 2) OS cultures began to from multilayered nodular structures as the apparent result of coalescing cellular aggeragates, and nearly all cells of nodurlar structures were cuboid (Fig. 1b).

By day 18, the cellular morphology in control cultures always remained spindle-shaped and did not

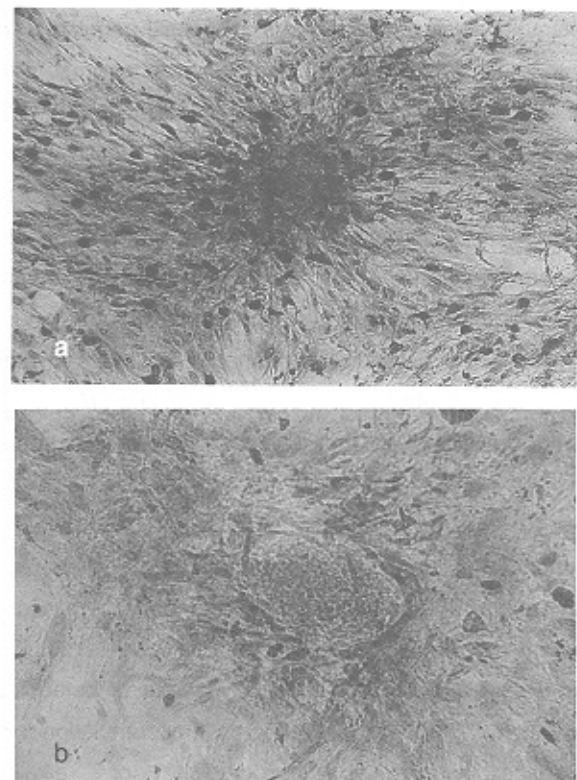


Fig 1: MSCs Cultured with os at day 6 (a), OS cultures at day 12 (b). (Light green staining, phase contrast microscopy, x100)

appear like MSCs grown in OS medium in which multilayered nodular structures were formed.

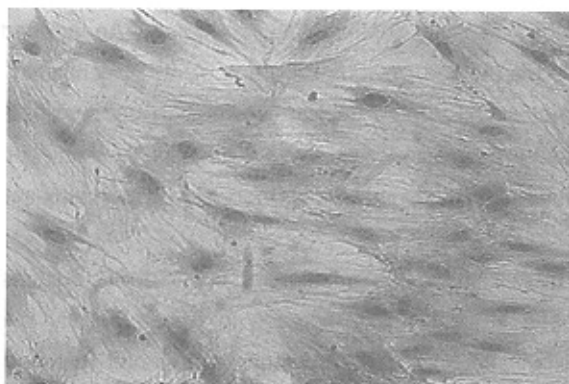


Fig. 2: Control cultures at day 12. (H&E staining, Phase contrast microscopy x100)

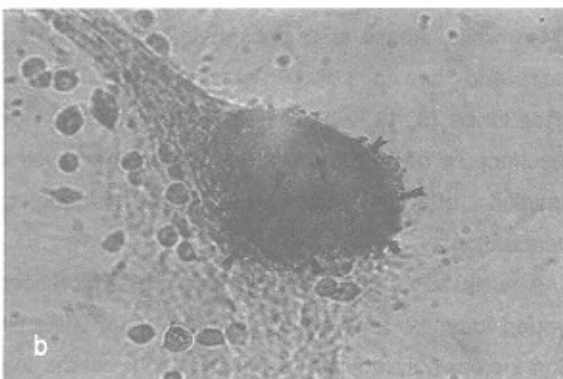
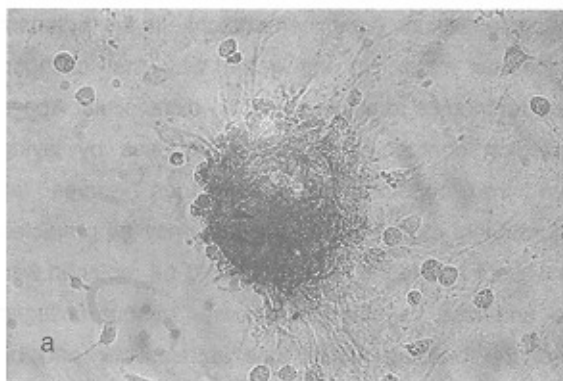


Fig. 3: Apase activity in OS cultures at day 6 (a) and at day 12 (b) demonstrated by fast red (Arrowheads). (Phase contrast microscopy x100)

#### \* Apase activity

Apase activity in OS cultures was elevated by day 6 and continued to climb until peaking at day 12, so by day 6, 197 (30-40%) and by day 12, 413 (over 80%) of stromal cell colonies were Apase-positive, when cultured in OS medium, while

control culture contained a few Apase activity (Fig. 3b and 4).

Apase activity in OS cultures declined between day 12 and 18, so by day 18 no Apase activity was detectable.

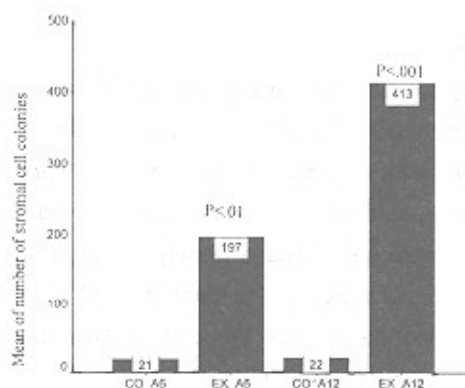


Fig. 4: Effect of 10 nM Dex on Apase expression in the stromal cell colonies

#### \* Mineralization

Early regions of mineralization were evident by day 12 in OS cultures by Von Kossa staining.

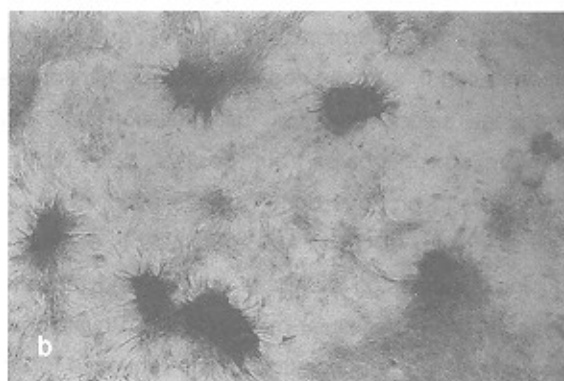
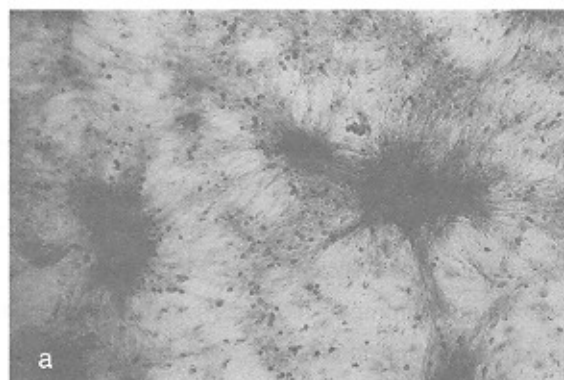


Fig. 5: Mineralization in OS cultures day 12 (a) and day 18 (b). (Von Kossa staining phase contrast microscopy, a;x100, b;x50)



(Fig. 5a), but never in cultures. Between day 12 and 18, mineral deposition increased.

By day 18, extensive mineralization occurred throughout the OS cultures (Fig. 5b). This mineralization localized to a few discrete foci.

## Discussion

During the 18 days assay period, MSCs cultured with optimize OS (100 nM Dex, 10 mM Na-β-GP and 50 μg/ml Asa), underwent a dramatic change in cellular morphology which was accompanied by a significant increase in Apase activity. During the latter half of this culture period, the deposition of a calcified matrix on the surface of the culture flask become evident by Von Kossa staining.

While osteoprogenitor cells are generally Apase-negative and those cells which are terminally differentiated; or osteocytic, are also weakly stained for Apase, the majority of cells in the middle of lineage progression are highly Apase-positive (16, 17, 18, 19, 20).

135 The addition of OS to MSC cultures caused a significant increase in Apase activity per cells over time, which we interpret to reflect the degree of progression into the osteoblastic lineage. The subsequent decrease in Apase activity per cell beyond day 12 correlates with advanced matrix mineralization and terminal osteogenic differentiation as MSCs become osteocytes. Rickard et al. found that treatment of rat marrow stroma-derived cells with Dex results in population of undifferentiated cells which retain the capacity for osteoblastic with secondary exposure to Dex (21). Furthermore, Turksen and Audiin demonstrated that Apase-negative osteoprogenitors are dependent upon Dex for differentiation into the bone-forming phenotype, but positive cells did not require Dex to produce bone nodules (22).

The addition of OS to MSCs cultures is capable of inducing expression of Apase activity, and formation of mineralized extracellular matrix (18,19). Physiological concentration of glucocorticoid were required for this phenomenon, which was further supported by an ascorbic acid (AsA) and Na-β-GP (18).

By contrast, cells grown in control medium without

OS, deposit a few mineral on the culture period as measured by Von Kossa staining.

Investigators have reported that Apase expression induced cellular differentiation, these Ap-induced changes include obvious morphological differentiation and clearly disrupted cellular proliferation because Apase expression exert a negative effect on cellular proliferation and cell locomtion by matrix-cell interaction (21).

In this study, the addition of OS to MSC cultures caused a signification increase in Apase activity per cell over time by day 12, which we interpret to reflect the degree of progression into the osteoblastic lineage.

As well as cellular differentiation, Apase expression on cell surface contributes to deposition of inorganic phosphate/calcium (i.e mineralization), in the adjacent extracellular matrix. In the same way that collagen fibers contributes to tissue elasticity, extracellular Apase expression contributes to tissue hardness by laying down the concrete, while collagen serves as reinforcement rods. From this model, may be predicted that without Apase the concrete would be replaced with mud and lose its strength. Likewise, without collagen fibers, the building would lose its elasticity and be brittle under stress (6).

Meanwhile, Apase is a phosphate/calcium binding protein and has a collagen-binding domain on its surface loops (8, 19, 20). Therefore, its expression on cell surface by day 12 in this study altered the physical properties of the membrane and leded to mineralization.

As well as Apase activity per cell decreased beyond day 12 and contemporary matrix mineralization increasingly advanced by day 18. It seems that this phenomenon is because of terminal osteogenic cell differentiation as MSCs was trapped in bone matrix and became osteocyte. Thereby, they gradually losed the Apase activity between day 12 and 18.

In conclusion the addition of osteogenic supplements (OS) to MSCs cultures induced Apase expression that contributes to cellular differentiation and mineralization of extracellular matrix.

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