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Apoptosis of rat osteoblasts in process of calcification *in vitro*

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KEY WORDS cultured cells; skull; osteoblasts; physiologic calcification; alkaline phosphatase; apoptosis

ABSTRACT

AIM: To establish a cell model of osteoblasts retaining their differentiated phenotype in culture and observe the apoptosis of osteoblasts in the process of calcification using a novel acetylcholinesterase (AChE) staining method. **METHODS:** Osteoblasts were isolated enzymatically from skull of newborn SD rats; alkaline phosphatase (AKP) activity was determined by reformed cobalt method and azo dye method; mineral deposition was assessed with Von Kossa staining and Fluo-3 staining; a novel AChE staining method was used to assay cellular apoptosis based on the higher expression of AChE in apoptotic cells. **RESULTS:** During the 44 d of cells cultured, primary rat skull-derived osteoblasts progressively developed into a bone-like tissue of multi-layered nodules of cells with mineralized extra-cellular matrix and the apoptotic cells increased while the matrix calcificated. **CONCLUSION:** The phenotype of developmental sequence of rat skull-derived osteoblasts can reflect the maturation of osteoblasts *in vitro*. It is a convenient model for the research of osteoblasts biology.

INTRODUCTION

Osteoblast, a highly biosynthetic cell type capable of complex process of matrix fibrillogenesis, as well as directing many of the activities of osteoclasts, plays an important role in bone deposition and remodeling[1]. Researches on osteoblastic differentiation have been done in the recent years[2,3]. McCarthy *et al*[2] investigated the possible presence of advanced glycation end product-binding proteins on osteoblast like cells, and found that rat and mouse osteoblast-like cells expressed specific advanced glycation endproduct-binding sites, with an affinity constant depending on the stage of osteoblastic differentiation. It suggested that the biological characteristics of osteoblast varied at different stages of differentiation, and might have clinical meaning.

Apoptosis is recognized as an important component of growth during embryogenesis, organogenesis, and tissue morphogenesis as well as in the maintenance of homeostasis in many adult tissues. The primary function of AChE is to hydrolyse ACh and thus terminates

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cholinergic neurotransmission[4]. However, recent studies have shown that this gene is also expressed in other cells when they are undergoing apoptosis, and postulated that AChE was induced and played an important role in apoptosis[5]. The AChE protein was found in the cytoplasma at the initiation of apoptosis and then accumulated in the nucleus. Because it is easy, cheap, rapid, and reliable, AChE cytochemical staining is employed to detect apoptotic cells which never express this enzyme while they are in viable condition[6,7].

To isolate osteoblasts that will retain in culture their differentiated phenotype, we derived cells from new born rat skull by sequentially timed-digestions with enzymes. The later digested cell populations express parameters having osteoblasts phenotype[8].

In this study, we studied the incidence of apoptosis in this cultured rat skull osteoblasts, with this novel staining method, based on the high expression of AChE in apoptoic cells. The purpose of this study was to confirm the role of this cell model in osteoblast research.

MATERIALS AND METHODS

Cell culture Osteoblasts were isolated mechanically from newborn rat skull as previously described[9, 10]. Briefly, skull (frontal and perietal bones) were dissected from Sprague-Dawley (SD) rats of 24-h old (Grade II, provided by Shanghai Research Center of Life Science, qualified certificate No 005, released from Animal Administration Committee of Chinese Academy of Sciences), endosteum and periosteum were stripped off, and the bone was cut into approximately 1-2 mm^2 pieces and digested with trypsin (2.5 g/L, 1:250, Gibco) and collagenase A (2.0 g/L, Sigma). The initial digestion including 20 min and 40 min digests was discarded. The third digestion lasted for another 60 min, and the cells were collected and cultured in small bottles containing a 8 mm×24mm cover glass with an initial seeding at a cell density of 1×10^7/L. The culture medium was changed every 3 d in all the experiments. After culture for 5-7 d in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10 % calf serum (Gibco), benzylpenicillin (1×10^5 U/L), and streptomycin (100 mg/L), cells reached confluence. The cultures were then supplemented with sodium β-glycerophosphate (10 mmol/L, Sigma) and ascorbate acid (50 mg/L, Shanghai Xinyi Pharmaceuticals Factory, China).

Histochemical analysis Cells attached to cover glasses were washed twice with phosphate buffer solution (PBS) on ice, fixed for 10 min with 2 % paraformaldehyde. Mineral deposition was assessed by staining cells with 3 % $AgNO_3$ for 30 min under bright light (Von Kossa staining)[11]. For alkaline phosphatase (AKP) detection, we used reformed cobalt method[12] and azo dye method (Shanghai Hongxiao Medical Reagents Institution Kits, China).

Fluo-3 staining Fluo-3/AM (Molecular Probes, 10 μmol/L) dissolved in Me_2SO was added to the cultures. After incubation for 30 min at 37 ºC, cells were washed 3 times by serum-free medium, and observed under the fluorescent microscope.

Identification of apoptotic cells A novel approach of apoptotic cell detection was utilized in the present study. Cells of 10 d, 20 d, and 40 d were first fixed (4 % paraformaldehyde in PBS, pH 7.4). Then they were stained by hematoxylin and observed under light microscope (Nikon, Japan). AChE cytochemical staining was performed according to the method of Karnovsky and Roots[13]. AChE activity was determined as described previously[14].

RESULTS

Morphology From d 2 to d 4 after primary culture, cells were polymorphological and most of them were in dividing phase. After 10-d culture, proliferation was decreased, regions of multilayered cells were apparent, and cells were surrounded by the extra-cellular matrix. By d 21, there were a lot of cell nodules in culture. Histochemical assays showed that there were very few AKP positive cells in the 2-4 d cultures (Fig 1A). However, since d 8 after isolation, cells exhibited heavier staining of AKP activity (Fig 1B, 1C). After d 15, Fluo-3 and Von Kossa staining of the minerized nodules showed hydroxyapatite deposition (Fig 2, 3).

Apoptosis Apoptosis is a naturally occurring suicide process exhibited in a variety of cell types. The

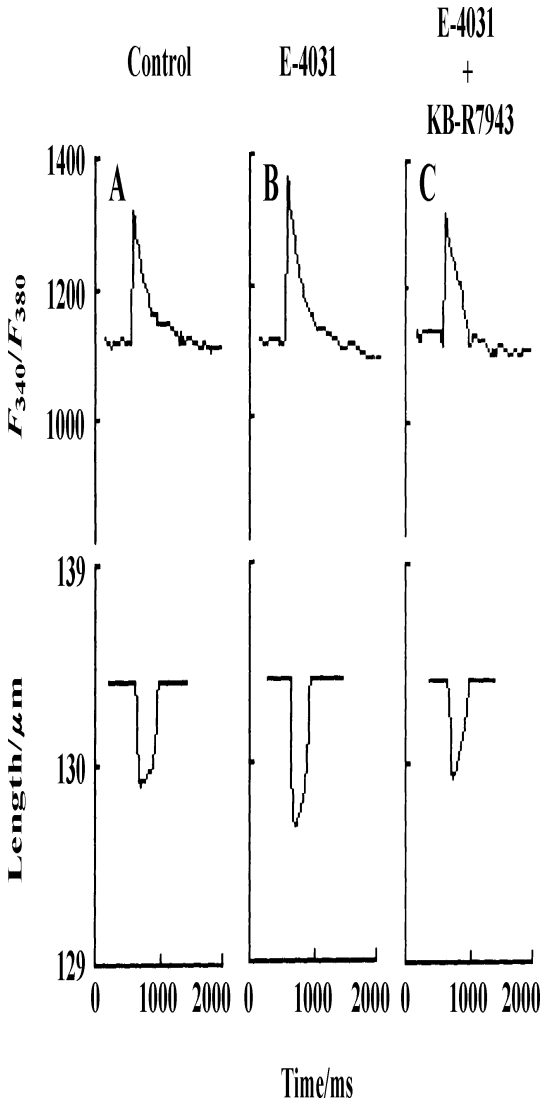

Fig 1. Osteoblasts isolated from rat skull. A) Three days after primary culture. B) Twelve days after primary culture. C) Eighteen days after primary culture. Azo dye staining, ×320.

differentiation of rat skull-derived osteoblasts *in vitro* progressed through three successive stages. During the proliferative stage, a few individual apoptotic cells were observed by AChE staining. Instead, we could find many cells undergoing mitotic division. As cell nodules were formed, proliferation was slowing down. With progressive development of the nodules and calcification of the extra-cellular matrix, a marked increase in the proportion of cells heavily expressed AChE was found (Fig 4A, 4B, 4C).

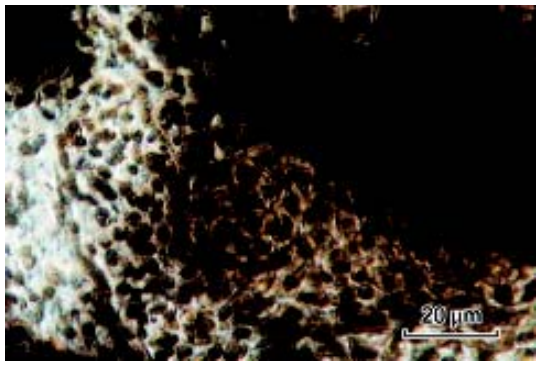

Fig 2. Osteoblasts isolated from rat skull 25 d after primary culture. Cobalt staining, ×320.

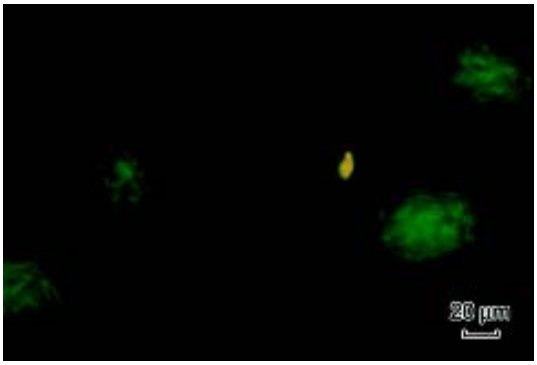

Fig 3. Osteoblasts isolated from rat skull 30 d after primary culture. Fluo-3 staining, ×100.

DISCUSSION

During a 44-d period, primary culture of rat skull-derived osteoblasts progressively developed into a bone tissue-like organization consisting of multi-layered nodules of cells with mineralized extra-cellular matrix. We found that there were a few AKP positive cells in 2-4 d after primary culture. Then the number of AKP positive cells increased, reflecting the enhanced AKP activity during the process of osteoblast maturation. Thus AKP positive cells appeared very early and existed throughout the process of osteoblast development. We have determined the AKP activity by BM/Keysys analyzer (unpublished data). The results showed that the activity of AKP remained elevated and reached the peak value during the stage of extra-cellular matrix formation (d 14-16), and decreased markedly after d 26. The AKP data taken together with the results of the his-

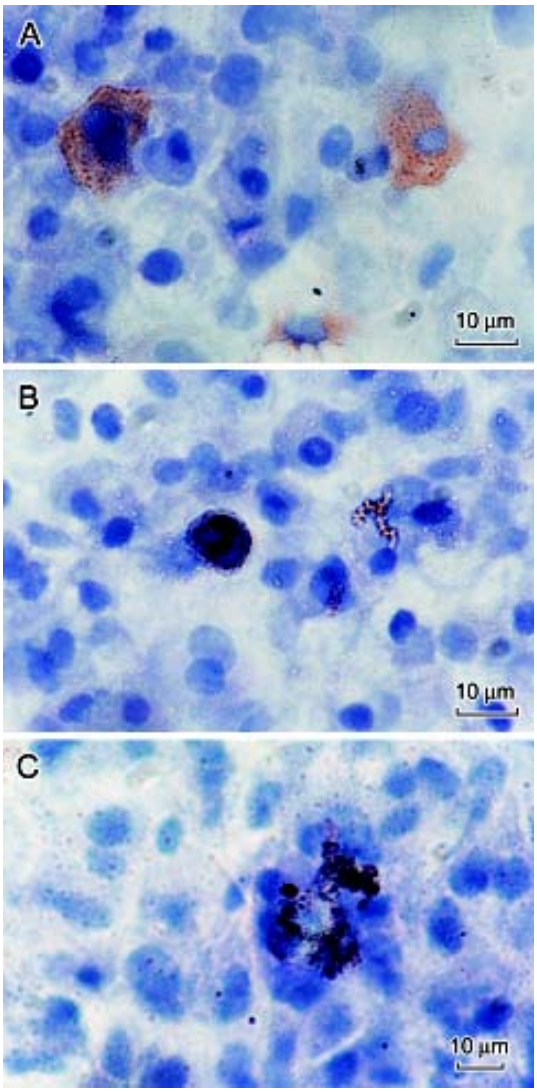

Fig 4. Osteoblasts isolated from rat skull 40 days after primary culture. A) Early stage of apoptosis, showing high AChE activity in cytoplasma (AChE staining, ×400). B) Mid stage of apoptosis, showing nucleus condensation (AChE staining, ×400). C) Final stage of apoptosis, showing cell debris and apoptotic bodies (AChE staining, ×320).

tochemical staining, we suggested that AKP should be regarded as an early marker of osteoblast differentiation.

Once osteoblasts have completed their bone-forming function, they are either entrapped in bone matrix, and become osteocytes or remain on the surface as lining cells. Nonetheless, 50 %-70 % of the osteoblasts initially present at the remodeling site can not be accounted for after enumeration of lining cells and osteocytes[15]. We hypothesize that the missing osteoblasts undergo apoptosis and our results supported this hypothesis with AChE staining method.

With enzymatic removel of extra-cellular matrix which was synthesized *in vivo*, we established an osteoblast model, in which the extra-cellular matrix that controlled the differentiation process of the surrounding cells, supported the reinitiation of the developmental sequence of proliferation and extra-cellular matrix maturation, mineralization, and apoptosis. This kind of culture could be maintained for up to 140 d with the cells and extra-cellular matrix retaining bone-like features. Therefore this rat skull derived osteoblast model described here is convenient for the research of osteoblast biology.

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体外培养的大鼠成骨细胞在钙化过程中的凋亡

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关键词 培养的细胞；颅骨；成骨细胞；生理性钙化；碱性磷酸酶；细胞凋亡

目的：建立在体外培养时能保持分化表型的成骨细胞实验模型，并观察成骨细胞在体外的凋亡. **方法：**用酶解的方法从新生 SD 大鼠颅骨分离成骨细胞；用改良钙钴法及偶氮染色法测定其碱性磷酸酶(AKP)活性；用 Von Kossa 染色法及 Fluo-3 染色法检测其钙化沉积物；根据凋亡细胞中乙酰胆碱酯酶(AChE)高表达的特性，用乙酰胆碱酯酶染色法观察不同时期的凋亡细胞. **结果：**原代大鼠颅骨成骨细胞在体外培养 44 天中，逐步形成包括多层细胞结节，钙化的细胞外基质组成的骨样组织，随着基质的钙化，凋亡细胞逐步增多. **结论：**大鼠颅骨成骨细胞在体外发育过程的表型能反映成骨细胞在体外的成熟过程，是研究成骨细胞生物学理想的实验模型.

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