行政院國家科學委員會專題研究計畫成果報告

計畫名稱：The development of in-vitro osteoclasts cell culture model by alveolar mononuclear cells

(英、中文) 以肺臟單核球體外培養蝕骨細胞之細胞培養模式之建立

計畫類別：☒ 個別型計畫 □ 整合型計畫

計畫編號：ＮＳＣ89－2314－B002－504 -

執行期間：89 年 08 月 01 日至 90 年 07 月 31 日

個別型計畫：計畫主持人：韓毅雄

共同主持人：孫瑞昇

整合型計畫：總計畫主持人：

子計畫主持人：

註： 整合型計畫總報告與子計畫成果報告請分開編印各成一冊，彙整一起繳送過國科會。

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執 行 單 位 ： 台 大 醫 學 院 骨 科

中 華 民 國 九 十 年 九 月 十 三 日

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英文：

The development of in-vitro osteoclasts cell culture model by alveolar mononuclear cells

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主持人：韓毅雄

中文摘要：

關鍵詞：肺臟單核球、骨母細胞、蝕骨細胞、體外培養

研究背景：

過去學者之研究已成功的證實蝕骨細胞可以經由骨髓或週邊血液之單核球所演化而來；本研究之主要目的在探討新生老鼠肺臟單核球與頭蓋骨骨母細胞體外共同培養後是否會演化為多核之蝕骨細胞．

方法：

本研究使用新生老鼠頭蓋骨利用酵素取得骨母細胞並自肺臟利用沖洗之方法取得單核球與頭蓋骨骨母細胞進行體外共同培養，以 DMEM medium**為培養基並進行培養**14**天後，觀察是否會演化為多核之蝕骨細胞．骨吸收作用則使用光學顯微鏡及掃描式電子顯微鏡加以觀察．**

結果：

肺臟單核球與頭蓋骨骨母細胞於維他命D3**存在下體外共同培養**14**天後，可行成含抗酒石酸作用之酸性磷酸脢之巨大多核細胞族群．骨吸收作用則在**7 - 14**天長期培養後可以觀察到．**

結論：

本實驗結果顯示，肺臟單核球可在體外適當之環境下經由骨母細胞作用形成巨大多核之蝕骨細胞．

英文摘要：

ABSTRACT

Background: Previous studies have shown that osteoclasts are derived from mononuclear cells of hemopoietic bone marrow and peripheral blood. The purpose of this study was to demonstrate the presence of multinucleated osteoclasts after adding mononuclear cells from alveolar mononuclear cells into new-born rat calvaria osteoblasts in vitro.

Methods: In order to utilize osteoclast-free bone, the fetal calvariae were obtained from newborn Wistar-rat calvaria and cultured in DMEM medium for 14 days. At day of osteoblasts culture, alveolar mononuclear cells were simultaneously isolated from the same rat with serial washing method and then co-cultured with the calvaria osteoblasts. Bone resorption characteristics were observed both with light microscope and scanning electron microscope examination.

Results: When alveoalr mononuclear cells were cultured for 14 days on the calvarial osteoblasts in response to 1 alpha, 25-dihydroxyvitamin D3, they formed colonies of tartrate-resistant acid phosphatase (TRAP)-positive mononuclear and multinucleated cells appeared in the colonies (TRAP-positive colonies). Resorption pits were seen in 7 - 14 days' long-term cultures.

Conclusions: These results indicate that osteoclasts can be derived from the mononuclear alveolar mononuclear cells to give rise to multi-nucleated osteoclasts in vitro when a suitable microenvironment is provided by calvarial osteoblasts cells.

Key words: Alveolar mononuclear cells, Osteoclasts, Osteoblasts, In vitro

INTRODUCTION

Bone is remodeled continuously during adulthood through the resorption of old bone by osteoclasts and the subsequent formation of new bone by osteoblasts. Under normal conditions, bone remodeling proceeds in cycles in which osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion (**Manolagas and Jilka 1995**). Osteoclasts are multinucleated cells with a specialized function to resorb calcified tissues (**Baron 1989, Suda et al. 1992**). The origin of the multinucleated osteoclast has been controversial, with osteoprogenitor cells (**Young, 1962**) and other cells of skeletal origin (**Toto and Magon, 1960**). Much evidence indicates that osteoclasts are probably derived from hemopoietic progenitor cells (**Owen 1978, Walker 1975**). The exact nature of osteoclast precursors and their differentiation process are still a matter of controversy (**Kurihara et al. 1990, Lee et al. 1991, Suda et al. 1992**). The purpose of this study is to demonstrate the formation of multi-nucleated osteoclasts after adding alveolar mononuclear cells into osteoblast-like cells from new-born rat calvariae osteoblasts. The combined methodologies of Turyna et al. (1996) and Wong and Cohn (1975) were used to determine the in vitro formation of osteoclasts.

MATERIALS AND METHODS

Osteoblasts Cell Culture

Sequential digestion of newborn Wistar-rat calvaria was performed by using a modification of the methods described by **Wong and Cohn (1975**). The cells released after treatment were immediately harvested by centrifugation and resuspended in culture medium.

Isolation of alveolar mononuclear cells

Same newborn Wistar-rats as described above were used. To obtain rat alveolar mononuclear cells, the isolated lungs were washed with 0.9% *(w/v)* NaCl through the trachea and the washings were pooled, centrifuged and the cell pellet resuspended in DMEM medium supplemented with 10% (v/v) heat-inactivated FBS (**Turyna et al. 1996**).

Histochemical Charactertization of Cultured Cells

Alveolar mononuclear cells obtained from lungs with a concentration of 5.0 x 10^5 were mixed with 1.0 x 10^4 calvarial osteoblastic cells per well in a 6-well plate. At 4 days, 7 days, 10 days and 14 days after culture, the dishes were fixed and stained for tartrate-resistant acid phosphatase (TRAP) by a commercially available assay kit (procedure no. 386, acid phosphatase, leukocyte, Sigma Co., Louis, MO, USA). The cellularity of experimental wells was determined by a MICD image analyzing system (MICD Software Series, Image Research Inc. Ontario, Canada).

Bone Resorption Assay

The isolated calvarial osteoblat-like cells and alveolar mononuclear cells were mixed, then placed on the bone slices in 6-well tissue culture plates and incubated at 37^{o}C under 5% CO_2 for 4 days, 7 days, 10 days and 14 days. At the end of the experiment, cell layers were removed by ultrasonication in 0.25M NH_4OH for 2 min. Resorption pits on the surface of bone wafer were then visualized using light and scanning microscopy.

Light microscopy

Bone samples were stained with 1% toluidine blue in 1% sodium borate for 5 min (**Boyde et al. 1985**), and resorption features detected in light microscopic

image were quantified using semiautomated image analysis. Image was obtained directly from an Olympus IM-2 microscope.

Scanning electron microscopy (SEM)

The bone slices were rinsed in phosphate-buffered saline (PBS) containing 0.2% ethylene-diaminetetra-acetic acid (EDTA) (Sigma Co., Louis, MO, USA), then placed in trypsin (0.4%) solution for five minutes to remove the layers of cells coating the bone surface. The bone slices were then washed vigorously in distilled water, dehydrated in graded alcohols, air-dried, critical point dried using $C0_2$ and subsequently sputter-coated with gold (Model IB-2 Ion Coater, Eiko Engineering, K.K. Japan). The resorption pits on each bone slice was observed by using an ISA ABT (model SX-30E, International Scientific Instruments, Inc., CA, USA) scanning electron microscope at an accelerating voltage of 20 kV.

RESULTS

Histochemical characteristics of isolated and cultured alveolar mononuclear cells

In long-term co-cultures of isolated alveolar mononuclear cells with osteoblast cells, more TRAP-positive cells were seen with a tendency to form clusters. That is, after alveolar mononuclear cells were co-cultured with rat-calvarial osteoblasts in the presence of $1,25(OH)_2D_3$ for 4, 7, 10 and 14 days, the cell counts of both osteoclasts and osteoblasts increased gradually; while the ratio of the osteoclasts to total bone cells remained constant (Table 1). However, scattered single TRAP positive cells were more frequently seen in the 4 days' culture, while large TRAP positive cell clusters were more frequently observed after 14 days' culture.

Cell cultures on bone slices and time course studies of alveolar mononuclear cells differentiation into osteoclasts-like bone resorbing cells

When alveolar monocytes were co-cultured with rat-calvarial osteoblasts for 1, 2, or 3 days, bone resorption was not observed. After 4 days of incubation, the bone slices still showed scanty evidence of bone resorption but this resorption consisted of small numbers of pits and which constituted smaller surface area of the bone surface. The proportion of bone slices showing evidence of bone resorption rose after 7 days' culture. Almost all bone slices showed evidence of bone resorption after 7 days of incubation; these consisted of large numbers of overlapping excavations on larger surface area of the bone slice.

SEM bone resorption assay

When calvarial osteoblasts co-cultures with alveolar mononuclear cells, bone slices showed many scattered resorption pits. The excavations ranged from simple, circular resorption pits of 5 μm in diameter to extensive but confluent areas up to 300 μm in diameter. In the areas of high-grade lacunar bone resorption, resorption pits composed of well-defined excavations with a fibrillar base of mineralised collagen fibres were seen. Resorption pits were seen frequently in 7 - 14 days' long-term cultures. They were not found in

4-day cultures.

DISCUSSION

In this study, we developed a new co-culture system to determine the origin of osteoclasts. When relatively small numbers of alveolar mononuclear cells were cultured for 14 days on the calvarial osteoblasts, tartrate-resistant acid phosphatase (TRAP)-positive mononuclear and multinucleated cells appeared in the colonies (TRAP-positive colonies) in response to 1-α, 25-dihydroxyvitamin D_3 at 4 days after co-culture of alveolar mononuclear cells and bone explants. It was the alveolar mononuclear cells, which were undoubtedly different from bone marrow stem cells, that gave rise to osteoclasts with the calvarial osteoblasts.

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