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A novel co-culture system for interaction of mouse osteoblasts and osteoclasts**☆

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Abstract

BACKGROUND: It is well known that bone remodeling is an important biological process in which osteoblasts and osteoclasts play the critical role. However, the detailed mechanisms of osteoblast-osteoclast communication in bone remodeling remain unclear.

OBJECTIVE: To *in vitro* establish a novel co-culture system for interaction of mouse osteoblasts and osteoclasts using the Transwell technology.

METHODS: Osteoblastic cell line MC3T3-E1 and preosteoclasts RAW264.7 were selected, and then the cells were induced to differentiate into osteoblasts and osteoclasts, respectively. The co-culture system for osteoblasts and osteoclasts interaction was established with Transwell co-culture plate (0.4 μm polyester film), and the osteoblasts and osteoclasts were co-cultured for 6 days. After that, the proliferation and differentiation activities of osteoblasts were analyzed through testing the cell activity and alkaline phosphatase activity, and the differentiation and bone resorption of osteoclasts were observed using tartrate-resistant acid phosphatase staining, toluidine blue staining, tartrate-resistant acid phosphatase activity detecting and scanning microscope technology.

RESULTS AND CONCLUSION: In the co-culture system, the unlimited proliferation ability of osteoblasts was decreased, while the differentiation activity was increased. Meanwhile, the preosteoclasts could differentiate into mature osteoclasts with bone resorption function. Thus, this co-culture system can be applied in the further study of osteoblast-osteoclast communication in bone remodeling.

Key Words: tissue construction; bone tissue construction; co-culture; osteoblasts; osteoclasts; alkaline phosphatase; tartrate-resistant acid phosphatase; National Natural Science Foundation of China

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INTRODUCTION

Osteoporosis, a metabolic bone disease, is harmful to human health. The main cause of osteoporosis is the imbalance between bone resorption and bone formation in bone remodeling, resulting in the loss of bone mass and osseous integrity[1]. Bone remodeling is a biological process to remove old bone and promote new bone formation and that is very important to bone metabolism. Mechanical loading plays an essential role in bone remodeling, because suitable mechanical stimulation can maintain normal bone volume, while removal of mechanical stimulation can cause the loss of bone mass[2].

Osteoblasts and osteoclasts are considered to be the key factors in bone remodeling and their interaction is regulated by a variety of mechanical and cytokine stimulations. Therefore, the study on the interaction between osteoblasts and osteoclasts is necessary to investigate the regulatory mechanism of bone remodeling. It is generally considered that co-cultured cells are significantly different from the cells cultured alone in their function. However, the co-culture

system of osteoblasts and osteoclasts has not been well established. Therefore, we established a novel co-culture system of osteoblasts and osteoclasts using transwell inserts, for investigating the proliferation and differentiation activity of osteoblasts and osteoclasts.

MATERIALS AND METHODS

Design

A cellular observation.

Time and setting

The experiment was performed at Institute of Medical Equipment of Academy of Military Medical Sciences from March 2011 to September 2011.

Materials

MC3T3-E1 cells and RAW264.7 cells were provided by Institute of Basic Medicine of Peking Union Medical College. Dulbecco minimum essential medium/F12 and fetal bovine serums were obtained from Hyclone Biotechnology. Toluidine blue and methylthiazolyldiphenyl-tetrazolium bromide were purchased from Tianjin Huashengyuan Company. The kits of alkaline phosphatase and tartrate-resistant acid phosphatase were purchased from Nanjing Jiancheng Company. 1α, 25$(OH)_2D_3$ and macrophage colony-stimulating factor were obtained from Peprotech Asia.

Methods

Establishment of co-culture system

MC3T3-E1 cells, a mouse monoclonal pre-osteoblastic cell line, were embedded with 5×10^4 on the bottom of the transwell plates, and RAW264.7 cells, a mouse pre-osteoclastic cell line, were incubated with 5×10^4 in the transwell inserts on the top of the MC3T3-E1 cells sheet. The co-culture system was maintained in Dulbecco minimum essential medium/F12 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 10^{-8} mol/L 1α,25$(OH)_2D_3$ and 50 ng/mL macrophage colony-stimulating factor in 5% CO_2 at 37 ℃. Simultaneously, MC3T3-E1 and RAW264.7 cells were cultured alone respectively for control. After co-cultured for 6 days, the sequential experiments were performed.

Cell viability assay for osteoblasts

Cell viability of osteoblasts was determined by methylthiazolyldiphenyl-tetrazolium bromide assay. Briefly, the methylthiazolyldiphenyl-tetrazolium bromide solution was added into each well of plates and then the cells were incubated in 5% CO_2 at 37 ℃ for 4 hours. Subsequently, the medium in each well was replaced with dimethyl sulfoxide to dissolve the methylthiazolyldiphenyl-tetrazolium bromide formazan. Finally, 20 μL solute per well was added to 96-well plates and quantified by the absorbance at 490 nm using an enzyme-labeled meter.

Alkaline phosphatase activity analysis for osteoblasts

Briefly, alkaline phosphatase activity in the cultured supernatant was determined on the base of the enzyme conversion of alkaline phosphatase. The activity of alkaline phosphatase was quantified by the absorbance at 520 nm according to the manufacturer's instructions.

Toluidine blue staining for osteoclasts

After fixed with 95% alcohol, the osteoclast-like cells on coverslips were stained in the 0.5% solution of toluidine blue at 56 ℃ for 20 minutes, and washed with distilled water. After dehydrate, clear and mount, multinuclear cells with the characteristics of abundant cytoplasm and vacuolation were observed as mature osteoclasts under a light microscope.

Tartrate-resistant acid phosphatase staining for osteoclasts

After fixed with 95% alcohol, the osteoclast-like cells on coverslips were incubated in the solution of naphthol AS-BI phosphate and tartrate at 37 ℃ for 60 minutes, followed by counterstaining with the haematoxylin solution. After dehydrate, clear and mount, the coverslips were observed and tartrate-resistant acid phosphatase-positive multinuclear cells were regarded as mature osteoclasts.

Tartrate-resistant acid phosphatase activity analysis for osteoclasts

Briefly, tartrate-resistant acid phosphatase activity in the cultured supernatant was detected by spectrophotometer on the base of the enzyme conversion of tartrate-resistant acid phosphatase. The activity of tartrate-resistant acid phosphatase was assayed by the absorbance at 530 nm according to the protocol.

Morphology of resorption lacunae observed under scanning electron microscope

First, the osteoclastic cells were wiped off the bone slices by ultrasonication with 0.25% ammonium hydroxide for 15 minutes. After rinsing in phosphate buffered solution, the bone slices were fixed with 2.5% glutaraldehyde and further in 1% osmium tetroxide solution. After dehydrated, air-dried and gold coated slices were examined in a scanning electron microscope for resorption pit.

Main outcome measures

Cell viability and alkaline phosphatase activity of osteoblasts, tartrate-resistant acid phosphatase/TB staining and tartrate-resistant acid phosphatase activity of osteoclasts, as well as bone resorption lacunae were determined.

Statistical analysis

All data were presented as means±standard deviation. Statistical differences were evaluated by one-way analysis of variance using SPSS 13.0 software and $P < 0.05$ was considered as significant.

RESULTS

Identification of osteoblasts in the co-culture system

Methylthiazolyldiphenyl-tetrazolium bromide assay: compared with the single culture group, the absorbance value of the osteoblasts in the co-culture group was decreased significantly ($P < 0.01$; Table 1).

Alkaline phosphatase testing: compared with the single culture group, the alkaline phosphatase activity of the osteoblasts in the co-culture group was increased significantly ($P < 0.01$; Table 1).

Table 1 Absorbance (*A*) value and alkaline phosphatase (ALP) activity of the osteoblasts in each group detected with methylthiazolyldiphenyl-tetrazolium bromide assay and ALP testing ($\bar{x}\pm s$, n=6)

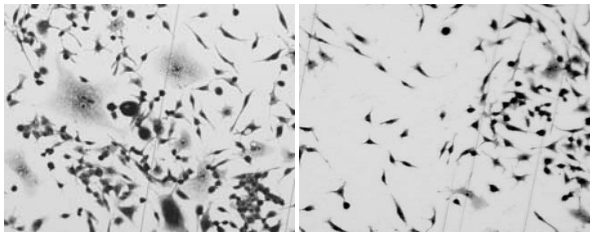
Group	*A* value	ALP activity (nkat/L)
Co-culture	0.304±0.004[a]	175.035±8.335[a]
Single culture	0.485±0.049	128.359±1.667

[a]$P < 0.01$, *vs.* single culture group

Identification of osteoclasts in the co-culture system

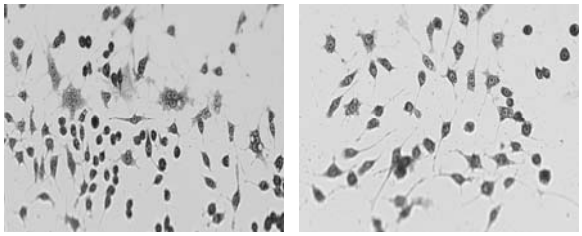
Tartrate-resistant acid phosphatase and toluidine blue staining: compared with the single culture group, many mature osteoclasts could be seen in the co-culture group, the size of the osteoclasts was increased with cytoplasm stretch and vacuolation, and many nucleuses could be seen (Figures 1, 2).

Tartrate-resistant acid phosphatase activity measurement: compared with the single culture group, the tartrate-resistant acid phosphatase activity of the osteoclasts in the co-culture group was increased significantly ($P < 0.01$; Table 2).



Co-culture group Single culture group

Figure 1 Tartrate-resistant acid phosphatase staining of osteoclasts in each group (×200)



Co-culture group Single culture group

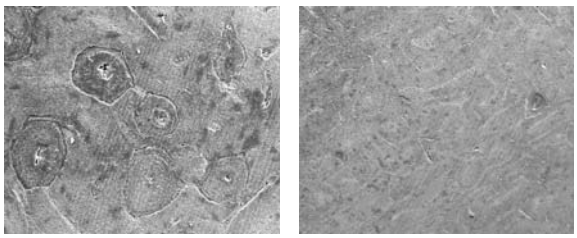
Figure 2 Toluidine blue staining of osteoclasts in each group (×200)

Table 2 Measurement of tartrate-resistant acid phosphatase activity (TRAP) of osteoclasts in each group ($\bar{x}\pm s$, n=6)

Group	TRAP activity (nkat/L)
Co-culture	21.07±3.62[a]
Single culture	6.77±1.13

[a]$P < 0.01$, *vs.* single culture group

Scanning electron microscope observation: compared with the single culture group, many round resorption pits which depressed significantly could be seen on the bone slices in the co-culture group, and had clear boundaries with the surrounding bone tissue (Figure 3).



Co-culture group Single culture group

Figure 3 Observation of osteoclasts in each group under scanning electron microscope (×400)

DISCUSSION

Osteoblasts and osteoclasts are the fundamental cells involved in bone formation and bone resorption. It has been proved that the balance between bone formation and resorption contributes to bone remodeling, which helps bone tissue to repair microdamages[3]. However, the detailed mechanisms of osteoblast-osteoclast communication in bone remodeling remain unclear. Researchers believe that a stable co-culture system for osteoblasts and osteoclasts is required for investigating the regulatory mechanism of bone remodeling.

At present, the co-culture system for different cells mainly includes two types: direct contact and indirect contact. It is defined as the direct contact that two types of cells are co-cultured directly by the synaptic contact for signal transduction[4]. Shi *et al* [5] established a direct contact co-culture model between osteoblasts and osteoclasts to study their interaction in function and structure. The disadvantage of this co-culture system is that the growth dominance of osteoblasts will cause the massive death of osteoclasts ultimately, which is unfavorable to long-term co-culture. Furthermore, we found it difficult to isolate a single type of cells from this co-culture system for molecular experiments. The type of indirect contact is well defined that two types of cells are co-cultured in the same condition without direct contact, and their interaction is dependent on numerous cytokines secreted from cells[6]. The popular system of indirect contact co-culture is a double-chamber cultivating mode with the advantages of free supernatant exchange without passage of cells, in which system two kinds of cells are both observed clearly under microscope[7]. Lu *et al* [8] used a metal mesh as the scaffold to establish an indirect contact co-culture model of osteoblasts and osteoclasts and found that their mRNA and proteins can be extracted in the different stages of cultivation. However, in this co-culture model, the osteoclasts in the metal mesh may fall off into the chamber of osteoblasts, resulting in the direct contact of the two cells. Thus, in the present study, transwell plates of 6-well size inserts (0.4 μm pores allowing only for passage of small soluble factors) were used to establish a novel system of indirect contact co-culture for osteoblasts and osteoclasts. In addition, mouse osteoblastic cell line MC3T3-E1 and mouse monocyte/macrophage cell line RAW264.7 have been shown to differentiate into mature osteoblasts and osteoclasts through the actions of several bioactive factors[9-10]. The results showed that in the co-culture system, the proliferation of osteoblast-like cells was attenuated, whereas the differentiation activity was promoted remarkably, suggesting they transformed into the mature osteoblasts. Simultaneously, the pre-osteoclastic cells were induced to differentiate into the mature osteoclasts with bone resorbing function in the co-culture system. Collectively, this stable co-culture system has been well established and can be applied in the study of osteoblast- osteoclast communication in bone remodeling.

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建立小鼠成骨与破骨细胞相互作用的共育新体系**☆

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文章亮点：

Transwell 共培养体系中成骨样细胞的无限增殖能力受到抑制，而其分化活性得到明显增强，提示其已向成熟的成骨细胞转化；共培养体系中还可见多个破骨前体细胞诱导分化为多核的成熟破骨细胞，并具有一定的骨吸收功能。

关键词：

组织构建；骨组织构建；共培养；成骨细胞；破骨细胞；碱性磷酸酶；抗酒石酸酸性磷酸酶；国家自然科学基金

摘要

背景：众所周知，骨重建是骨组织中重要的生物学反应过程，其中成骨细胞与破骨细胞发挥了关键作用。但目前，关于骨重建中成骨与破骨细胞间信号传递的深层机制还不清楚。

目的：利用 transwell 技术，在体外建立一种成骨与破骨细胞的新型共育体系，为深入研究骨重建中成骨与破骨细胞的相互作用提供成熟的实验模型。

方法：采用 MC3T3-E1 成骨样细胞株与 RAW264.7 破骨前体细胞株，进行体外成骨与破骨细胞的诱导分化，并利用 Transwell 共培养板(0.4 μm 聚酯膜)建立成骨与破骨细胞的共育体系。共培养 6 d 后，通过测定细胞活性和碱性磷酸酶(ALP)活力分析成骨细胞的增殖和分化活性，利用抗酒石酸酸性磷酸酶(TRAP)染色、甲苯胺蓝(TB)染色、TRAP 活性测定及扫描电镜技术观察破骨细胞的分化及骨吸收功能。

结果与结论：共培养体系中成骨样细胞的无限增殖能力减弱，而分化活性明显增强，同时破骨前体细胞被诱导分化为成熟的破骨细胞，并具有一定的骨吸收功能。因此，该共培养体系可用于骨重建中成骨与破骨细胞间信号通路的深层研究。

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