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Effect of Fetal Bovine Serum on Osteoclast Formation in vitro

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Abstract: Osteoclasts, the bone-resorbing cells, are unique multinucleated cells which show the ability to destroy the bone tissue through dissolution of hydroxyapatite and degradation of organic matrix components. Fetal Bovine Serum (FBS) contains a large number of nutritional and macromolecular factors, it’s widely used as a cell culture supplement. Previous studies have found that FBS is essential for cell growth and it is also a potent inducer of osteoclast formation. However, relatively little is known regarding the mechanism of this effect. The aim of this study was to further explore the potential role of FBS on osteoclast formation and its mechanism. RAW 264.7 cells were cultured in medium with different FBS concentrations (10 %, 5 %, 1 %). The experiments were designed and performed including Tartrate-resistant acid phosphatase (TRAP) staining, Immunostaining, Transwell migration assay, Real time polymerase chain reaction (RT-PCR), and Western blot analysis. The results demonstrated that FBS promoted osteoclastogenesis in suitable concentrations by regulating migration of osteoclast precursors and expressions of TRAP and Cathepsin K.

Keywords: Fetal Bovine Serum (FBS), Osteoclast, Tartrate-resistant acid phosphatase (TRAP), Cathepsin K, RAW264.7 cell

Introduction

Osteoclasts are the principal bone-resorbing cells, the balance between osteoclasts and osteoblasts has a profound impact on skeletal homeostasis. Disorders of skeletal balance, such as osteoporosis, are typically induced by excess bone resorption rather than bone formation. Osteoclast precursors (OCPs) originate from monocyte/macrophage-lineage hematopoietic cells and usually exist in bone marrow cavity and blood stream1,2). They circulate systemically, proliferate, migrate into the bone microenvironment and attach onto the bone surfaces targeted for bone resorption activity. OCPs migrate toward bone-lining osteoblasts possessing Receptor Activator of NF-κB Ligand (RANKL) on their surface, which is critical for osteoclast differentiation. Tartrate-Resistant Acid Phosphatase (TRAP) is an iron-binding protein, which is highly expressed in osteoclasts. It has been suggested that TRAP participates in specialized electro-chemical reactions associated with osteoclast related bone matrix resorption3). Expression of TRAP is reported to be associated with the activation and differentiation of osteoclasts, the concentration of which in serum is also utilized as a biochemical marker of osteoclast function and bone resorption activity. Cathepsin K is a cysteine protease secreted by osteoclasts, which is essential for degradation of matrix collagen and activation of TRAP during bone resorption4,5). It is reported that global deletion of Cathepsin K in mice decreases bone resorption, which leads to osteopetrosis6).

Fetal bovine serum (FBS) is the sterile liquid that is obtained from the clotted blood of the bovine fetus, which contains numerous factors that are needed for the survival and propagation of mammalian cells in culture7,8). Despite advances in the fabrication of standardized serum-free media over the last decades9), FBS still remains the most widely used cell culture medium supplement in cell biology. Normally for most mammalian cells, 10 % FBS is applied into the medium to provide survival factors and nutrition. FBS is a potent inducer of osteoclast formation10). Pervious study has demonstrated that osteoclast formation is serum dose-dependent11). However, the mechanism of this effect is not well explained. We found that FBS promoted osteoclastogenesis in suitable concentrations by regulating migration of OCPs and expressions of TRAP and Cathepsin K.

Materials and Methods

Cell culture

RAW264.7 cells (Sigma, St. Louis, MO, USA) were cultured in α-MEM containing 100 U/ml penicillin, 100 U/ml streptomycin and different concentrations of FBS (10 %, 5 %, 1 %) (Sigma, St. Louis, MO, USA) separately. Cells were incubated in a humidified incubator at 37°C and 5% CO₂. Medium was replaced every two days.
Osteoclast formation

RAW264.7 cells in different FBS concentrations as described above were cultured for 4 days in presence of soluble RANKL (100 ng/ml) (R&D System, Minneapolis, MN, USA). Then cells were fixed with 4% formaldehyde and stained with TRAP and F-actin. TRAP+ multinuclear osteoclasts with greater than 3 nuclei per osteoclast. Osteoclast area was measured in F-actin immunostaining pictures.

Table 1. Primer Sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward(5'-3')</th>
<th>Reverse(5'-3')</th>
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<tbody>
<tr>
<td>TRAP</td>
<td>AGGACGTGTTCTCTGACCG</td>
<td>CGCAAACGGTAGTAAAGGG</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>CGAAAAGAGCTGCTCGCAACA</td>
<td>TGGGTAGCAGCAGAAACTTG</td>
</tr>
<tr>
<td>L32</td>
<td>GAGCTGCTCAAACGGCAAAC</td>
<td>TGGACGGCTATGCTGGT</td>
</tr>
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Figure 1. FBS induces osteoclast formation at suitable concentrations. (A) The osteoclast formation in different concentrations of FBS stained by TRAP and F-actin. a,d: 10% FBS; b,e: 5% FBS; c,f: 1% FBS (10X). Scale bar, 150 μm. (B) TRAP+ multinucleated osteoclasts with greater than 3 nuclei per osteoclast. (C) Mean area per osteoclast. Each value represents the mean ± SEM of three independent measurements. * indicate significant decrease compared with 10% FBS group (P < 0.05).

Figure 2. FBS promotes migration of RAW264.7 cells at suitable concentrations. RAW264.7 cells migration was examined by a transwell assay. Each value represents the mean ± SEM of three independent measurements. * indicate significant decrease compared with blank group (P < 0.05).

Transwell migration assay

Chemotactic experiment was measured in polycarbonate filter, 8-im size transwell chambers (Corning, Corning, NY, USA). The upper chamber included 10^5/well RAW264.7 cells,
while the lower chamber contained the same medium with M-CSF (10μg/ml) (R&D System, Minneapolis, MN, USA) and different concentrations of FBS (10%, 5%, 1%). Wells without FBS were set as blanks. Cells were cultured in a humidified incubator at 37°C and 5% CO2. After 6 hrs, the membrane was removed and slides were mounted with Fluoroshield containing DAPI. RAW264.7 cells that had migrated to the bottom chamber were counted by fluorescence microscopy.

**RNA isolation and real time polymerase chain reaction (RT-PCR)**

Total RNA was extracted from RAW264.7 cells that were cultured with RANKL for 3 days as described above using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). Complementary DNA (cDNA) was produced using a transcriptase PCR kit (ReverTra Dash, Toyobo Biochemicals, Osaka, Japan). The relative mRNA levels were determined by RT-PCR with primers and labeled probes. The primers used are presented in Table 1.

**Western blot analysis**

RAW264.7 cells with RANKL treatment as described above were washed with cold PBS and lysed in cold Tris-HCL (50 mM, pH=7.4), 10mM EDTA, 4.3M urea and 1 % Triton X-100. Proteins were subjected to SDS-PAGE using 10% separation gel and transferred to a nitrocellulose membrane. The membrane was then blocked for 2hrs at room temperature with 5% bovine serum albumin in TBST solution (10 mM Tris-HCl, pH=8.0; 150mM NaCl; 0.05 % Tween-20). Subsequently, the blots were incubated with the corresponding primary antibodies (rabbit anti-TRAP, rabbit anti-Cathepsin K) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in the TBST solution overnight at 4°C, followed by 2hrs incubation with secondary goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), conjugated with horseradish peroxidase, and visualized with an enhanced luminol-based chemiluminescent (ECL) kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The OD of the bands was quantified using LAS-1000 luminescent image analyzer software (Fujifilm, Berlin, Germany).

**Statistical analysis**

One-way analysis of variance and Tukey’s multiple comparison tests were performed to detect any significant effects that occurred as a result of the experimental variables. All results are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**FBS promoted osteoclast formation in suitable concentrations**

To assess the effect of FBS on osteoclast formation, RAW264.7 cells were stimulated with RANKL in culture medium containing different concentrations of FBS (10 %, 5 %, 1%). As demonstrated in Fig. 1A, FBS induced osteoclast formation in medium with 5% and 10% FBS, while the osteoclastogenesis was inhibited in 1% FBS groups. There are 78% and 82% decrease separately in the number of TRAP+ multinucleated cells (Fig. 1B) and the average

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**Figure 3. FBS regulates target genes during osteoclastogenesis.** (A) Relative fold changes of TRAP and Cathepsin K mRNA expression levels measured by RT-PCR in RAW264.7 cells cultured by 10% FBS, 5% FBS, and 1% FBS separately. (B) Protein levels of TRAP and Cathepsin K measured by western blot in RAW264.7 cells. Each value represents the mean ± SEM of three independent measurements. * indicate significant decrease compared with 10% FBS group (P < 0.05).
area per osteoclast in 1 % FBS groups (Fig. 1C) compared to 10% groups, whereas no difference was observed between 10 % and 5 % FBS groups.

**Effect of FBS on migration**

M-CSF stimulated a 3.8-fold and 4.5-fold increase of migrated cell number above baseline in 5% and 10% FBS groups respectively, however, there was no difference between blank and 1% FBS groups (Fig. 2). These results have suggested that FBS promotes OCPs migration, which is inhibited in a lower FBS concentration.

**Effect of FBS on gene and protein expression**

The examination of TRAP and Cathepsin K expression was carried out by RT-PCR and western blot. In 1 % FBS groups, the mRNA level of TRAP and Cathepsin K decreased by 53 % and 40 % separately compared to 10 % FBS groups, whereas no difference was observed between 10% and 5 % FBS groups (Fig. 3A). As showed in Fig. 3B, the protein levels of TRAP and Cathepsin K were also reduced in 1 %FBS groups, and there was only slight decrease in 5 % FBS groups compared with 10 % FBS groups. This result suggested that FBS may promote osteoclastogenesis by regulating TRAP and Cathepsin K.

**Discussion**

Osteoclasts are multinucleated cells that derive from hematopoietic progenitors in the bone marrow or blood system. They are the only cell type in the body that major in bone resorption and play a critical role in keeping normal skeletal homeostasis (growth and modeling), maintaining bone integrity, and calcium metabolism. The decreased osteoclast activity, which causes increased bone mass (osteopetrosis), or the increased osteoclast activity, which induces decreased bone mass (osteoporosis), will both lead to bone disorders. FBS is an essential medium supplement in culturing OCPs, it is also a potent inducer of osteoclast-like cell formation and its concentration plays an important role in osteoclastogenesis. Our results have demonstrated that FBS promotes osteoclast formation in suitable concentrations, such as 5% and 10%, while the osteoclastogenesis is inhibited when FBS is reduced to 1 %. The osteoclastogenesis exhibits no difference between 5 % and 10 % FBS groups, which suggests that there is a platform for osteoclast formation with FBS concentrations during this range, and the formation process will be inhibited if a lower concentration than this platform is applied.

OCPs migration ability is one of key attributes in osteoclastogenesis[2], which is essential for OCPs to attach onto the bone surface, fuse with each other, and mediate bone resorption. It involves in release of these cells into the circulating system from bone marrow and mobilizing from the blood stream to peripheral tissues, where OCPs can differentiate into mature osteoclast and are capable of expressing specific markers of osteoclast such as TRAP, Cathepsin K, etc and resorbing bone. The mobilization of OCPs requires a wide range of molecules and cytokines, which may potently stimulate the chemotactic recruitment and differentiation of OCPs[13]. FBS is the liquid fraction of clotted blood from fetal calf, which contains a large number of nutritional and macromolecular factors essential for cell growth[14]. Our results showed that 10% and 5% FBS in cell culture medium promoted the migration of OCPs, while 1 % FBS inhibited this processes, which suggested that some growth factors or molecules in FBS might control the osteoclast formation by regulating the migration of OCPs. TRAP and Cathepsin K are two of the proteins that have been associated with osteoclast formation and function. TRAP is an iron-containing enzyme that is found in human and murine species, which distributes in diverse tissues including bone and cartilage[15]. TRAP activity is regarded as an important chemical marker of osteoclasts and has shown to be essential for skeleton formation. It is reported that TRAP deficiency leads to a decreased resorptive activity during endochondral ossification, which results in an osteopetrotic phenotype and shortened long bones in adult mice[16-19]. TRAP also plays an essential role in osteoclast migration, it is suggested that osteoclast migration is regulated in part by TRAP-mediated dephosphorylation of osteopontin[19]. Cathepsin K, a lysosomal cysteine protease, exerts strong elastinolytic and collagenolytic activity[20]. It can easily degrade type I collagen, the major component of the organic bone matrix and has been suggested to be responsible for the proteolytic activation of TRAP[21]. Our results suggested that FBS regulates both the mRNA and protein expressions of TRAP and Cathepsin K in suitable concentrations, while the regulation will be inhibited once the concentration reduced to a certain extent.

The results of the present study have demonstrated that FBS promotes osteoclast formation by favoring OCPs migration at normal and suitable culturing concentrations and regulating expressions of TRAP and Cathepsin K, whereas a lower concentration turns out to play an inhibitory role. FBS contains numerous factors that are needed for survival of cells, therefore, it is promising to explore further what moleculars or factors contained in FBS control this process. These factors will be promising candidates for the treatment of patients with osteoporosis and other bone-resorbing diseases.

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