不同大小焦二磷酸鈣顆粒對蝕骨細胞之影響

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一、中文摘要

骨組織損傷。不論是由於外傷、腫瘤、或是感染引起，只要其造成骨骼缺損即須使用適當物質以促進骨骼之癒合。但是自體骨移植或異體骨移植均有其使用上之缺點。近年來由於在生醫材料上的進步，使得骨科手術亦有長足的進展。過去幾年來，本實驗室已成功地證實焦二磷酸鈣在植入體內後其生物適應性極佳且可以逐漸為生物體所解離。但是解離後焦二磷酸鈣顆粒是否會影響生物體，則尚未有適當之研究。本研究之主要目地即在測定各種不同顆粒大小焦二磷酸鈣對蝕骨細胞之影響。

首先將大白鼠骨母細胞以 EDTA-Trypsin 打下後以 1 x 10^5 cells/well 濃度置於 3.0 x 3.0 cm 培養盤中。以 DMED + 10% FBS + penicilline & streptomycin 於 37°C 以 5% CO_2 培養 24h 以促進蝕骨細胞之貼附。之後 0.1%(ww)四種不同顆粒大小焦二磷酸鈣(0.5 - 3.0 μm, 37 - 63 μm, 177 - 250 μm, and 420 - 841 μm)與 DMEM + ITS 之混合培養液一同培養。於 3h、1、3、7 days 分別取出培養液，以 500μl 分裝入 eppendorff 中，冰凍於零下 80°C。並對細胞數、TGF-β1 (Transforming Growth Factor-β1)、PGE_2 (Prostaglandin E_2)、ALP (Alkaline Phosphatase)等加以測量。並以 ANOVA (Analysis of Variances)統計方法分析，當 P 值小於 0.05 時定義為具有統計學意義。

經由此實驗得知在小顆粒之焦二磷酸鈣(<53 μm)對蝕骨骨細胞與骨母細胞均有明顯下降，而在大顆粒之焦二磷酸鈣(>177 μm)骨母細胞明顯上升而蝕骨骨細胞則仍是有明顯下降現象。同時細胞內及培養基中之 prostaglandin E_2 有明顯上升之趨勢。經由本研究焦二磷酸鈣磷酸鈣與骨母細胞間之生化及分子關係可以得到釐清。而焦二磷酸鈣是否具有臨床使用的潛力也可以獲得確定。

關鍵詞：焦二磷酸鈣、顆粒大小、前列腺素、胞動素、骨母細胞。

Abstract

An *in-vitro* bone-cell culture model was used to evaluate the potential application of sintered β-dicalcium pyrophosphate (SDCP) in arthroplasty surgery. Primary osteoclasts/osteoblasts were co-cultured with different sizes of SDCP particles. The changes in cell counts and the synthesis and secretion of alkaline phosphatase, acid phosphatase and prostaglandin E_2 in response to the SDCP particles were monitored. When bone cells were cultured with SDCP particles smaller than 53 μm, both the osteoblast and osteoclast cell counts decreased significantly. When the SDCP particles were larger than 177 μm, while the osteoblast population increased significantly, the osteoclast population decreased significantly. Simultaneously, the titer of prostaglandin E_2 in the medium and the cytoplasmic prostaglandin E_2 increased significantly. It is concluded that SDCP is a potentially useful bioceramic for the prevention of osteoclast-mediated periprosthetic osteolysis.

Keywords: sintered dicalcium pyrophosphate, particle size, osteoclasts

Introduction

With advances in ceramics technology, the application of calcium phosphate bioceramics as a bone substitute has recently received considerable attention. During the last decade, a large number of biomaterials have been proposed as artificial bone filers for repairing bone defects. The material that has been most widely used in clinical medicine is hydroxyapatite (HA) ceramics. The use of block forms of porous HA has resulted in an unacceptable high failure rate in clinical applications. As an intermediate product in the biological mineralization process, sintered β-dicalcium pyrophosphate (SDCP, formula β-$Ca_2P_2O_7$) has been proved to have great potential as an *in vivo* biodegradable bone substitute (**Lin et al. 1995, Lin et al. 1996**). However, the effect of SDCP particles on the bone cells was not clear in that study. In order to quantify the effect of particle size on bone cell metabolism, an osteoclast cell culture model system was set up in the presence of SDCP particles in the presence of various sized particles. The aim of this investigation was to study the possible effects and identify the mechanism by which the various sizes of SDCP particles and the osteoclasts interacted. This would lead to an evaluation of the possible clinical application of SDCP bioceramics in arthroplasty surgery.

Materials and Methods

The rat spleen-calvaria co-culture system was performed as described by Tanaka et al. (**Tanaka et al. 1993**). The medium surrounding a confluent rat bone cells culture was removed and the cells washed twice with PBS solution. Dulbecco's modified Eagle's medium supplemented with ITS (Insulin, 10 μg/ml medium; Transferrin, 5 μg/ml medium and sodium selenite 5 x 10^{-3} μg/ml medium; Sigma, St. Louis, MO, USA) was mixed with 0.1% (1 mg/ ml) of the various size SDCP particles and added to the cells. The day of addition of the new medium was considered to be the zero day of culture. The test medium was removed from wells at 3 hours, 1 day, 3 days, and 7 days,

aliquoted into 500 µl batches and then frozen at -80°C for further analysis.

At the end of the experiment, cells in the wells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available assay kit (procedure no. 386, acid phosphatase, leukocyte, Sigma Co., St. Louis, MO, USA). Briefly, cells were fixed with citrate/acetone solution for 30 seconds at room temperature. The fixed cells were incubated in the dark with a staining mixture for 1 hour at 37°C. The cells were washed with deionized water for 3 minutes, counter-stained with acid hematoxylin, and then observed by inverted microscopy (Olympus, IMT-II, Hatagaya, Shibuya-ku, Tokyo, Japan). The cell counts of the control and experimental wells were determined by a MICD image analyzing system (MICD Software Series, Image Research Inc., Catharine, Ontario, Canada). The mean osteoclast cell and non-osteoclast cells (i.e. osteoblasts) populations in four randomly selected high-power fields (0.06 mm^2) were measured by counting the number of nuclei caught by a CCD-72 camera (Dage-MII Inc., Michigan, Indiana, USA) through the microscope. The cell population per unit area (i.e. per mm^2) was measured as a mean of these four measurements. The total cell count per well was calculated by multiplying to obtain the count for the area of each well (940 mm^2/well). The cellular responses of treated sample were compared with control samples, and expressed as a percentage of the control values.

Alkaline phosphatase (ALP), acid phosphatase (ACP) activity, and prostaglandin E_2 (PGE_2) released from the cells into the medium and their intracellular content were measured using a commercially available assay kit.

Results and Discussion

As shown by this study, the bone cell counts changed significantly after adding SDCP particles. If the particle size was larger than 177µm, there was a significant increase in the osteoblast cell population; while the osteoclast cell population decreased significantly. When represented as a percentage of the control cell counts, the osteoblast counts increased by 140.1 - 153.8 % compared to the control counts for a SDCP particles size larger than 177 µm and osteoclast counts decreased by 48.0 – 82.6 % compared to the control counts. The osteoblast and osteoclast cell population decreased significantly when SDCP particles were smaller than 53 µm. When bone cells were cultured with SDCP particles smaller than 53 µm, the osteoblast counts decreased by 41.7 – 54.3 % compared to the control counts for a SDCP particles size smaller than 53 µm and the osteoclasts decreased by 1.8 – 24.8 % compared to the control counts.

While the larger SDCP particles may limit osteolysis, smaller particles may be generated in patient and the newly-generated smaller SDCP particles will compromise the osteoblasts as well as osteoclats. As shown above, a decrease in cell number after exposure to the test material could be used as a marker for toxicity. It has been suggested that toxicity due to direct contact of the particles with the cells only occurs with particles smaller than about 5 µm in diameter. The decrease in cell population was more obvious in the osteoclast counts. This result suggests that the rate of osteoclast proliferation and/or differentiation decrease significantly after the addition of the SDCP particles into the culture.

The changes in ALP and ACP secretions with the various sizes of SDCP particles did not attain a statistically significant level. However, the cytoplasmic ALP and ACP titer for various size SDCP particles decreased significantly. The changes in cytoplasmic ALP and ACP titer was more evident when bone cells were cultured with the smaller SDCP particles. This findings is in agreement with the changes in the cell counts of the bone cells.

The increase in PGE_2 titer in the culture medium and the cytoplasm of bone cells when cultured with the smaller size SDCP particles were quite obvious. The PGE_2 titers increased by 2.39 to 3.13 times compared to the control. It had been reported that PGE_2 inhibits osteoclast differentiation from precursor cells (**Quinn et al. 1997**). The smaller size SDCP particles thus could affect osteoclast cells differentiation by the PGE_2 pathway. There could be an inhibition of osteoclastic recruitment to the bone surface and an inhibition of the activity of osteoclasts that have reached the surface, and this could shorten the life span of the osteoclasts, perhaps by inducing apoptosis. The changes in osteoclast cell counts and the ratio of osteoclasts to osteoblasts after adding the SDCP particles to the bone cells cultures are possibly mediated by the PGE_2 pathways.

Peri-prosthetic osteolysis leading to aseptic loosening is a major cause of failure in total joint arthroplasty. The process of osteolysis begins with the generation of implant material wear particles. These particles stimulate macrophages and giant cells, which release mediators that stimulate the osteoclasts, leading to peri-prosthetic bone resorption. A net loss of bone at the tissue-implant interface thus occurs via an increase in bone resorption by osteoclasts and/or via a decrease in bone formation by osteoblasts. A bioceramics can contribute to bony growth and incorporation by inhibiting the functions of osteoclasts or accelerating the function of osteoblasts. The results of this study show clearly that larger sized SDCP particles are potentially useful for the prevention of osteoclast-mediated periprosthetic osteolysis. Further *in vivo* and clinical studies are required to further analyze the effects of SDCP.

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