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Short communication

Sustained release of small molecules from carbon nanotube-reinforced monetite calcium phosphate cement

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ABSTRACT

The interest in developing calcium phosphate cement (CPC) as a drug delivery system has risen because of its capability to achieve local and controlled treatment to the site of the bone disease. The purpose of this study was to investigate the release pattern of drug-carrying carboxylic acid-functionalized multi-walled carbon nanotube (MWCNT)-reinforced monetite (DCPA, $CaHPO_4$)-based CPC. Z-Leu-Leu-Leu-al (MG132), a small peptide molecule inhibiting NF-κB-mediated osteoclastic resorption, was used as a model drug. MG132 was added into the cement during setting and released into the medium used to culture indicator cells. Significant cell death was observed in osteoblast MC3T3-E1 cells cultured in the medium incubated with MG132-loaded CPC; however, with the presence of MWCNTs in the cement, the toxic effect was not detectable. NF-κB activation was quantified using a NF-κB promoter-driving luciferase reporter in human embryonic kidney 293 cells. The medium collected after incubation with drug-incorporated CPC with or without MWCNT inhibited TNFα-induced NF-κB activation indicating that the effective amount of MG132 was released. CPC/drug complex showed a rapid release within 24 h whereas incorporation of MWCNTs attenuated this burst release effect. In addition, suppression of TNFα-induced osteoclast differentiation in RAW 264.7 cell culture also confirmed the sustained release of MWCNT/CPC/drug. Our data demonstrated the drug delivery capability of this cement composite, which can potentially be used to carry therapeutic molecules to improve bone regeneration in conjunction with its fracture stabilizing function. Furthermore, it suggested a novel approach to lessen the burst release effect of the CPC-based drug delivery system by incorporating functionalized MWCNTs.

1. Introduction

Intensive efforts have been made over decades seeking suitable materials for bone augmentation. Due to the desirable handling properties as well as excellent biocompatibility and osteoconductivity, calcium phosphate-based cements (CPCs) have been considered as a promising candidate for use in orthopedic surgery [1]. The interest of developing CPCs as a drug delivery system has also risen because of its capability to achieve local and controlled treatment to the site of the diseases. During preparation of CPCs, drugs can be incorporated through either the solid (powder) phase or liquid phase. Alternatively, drugs can also be loaded after setting [2,3]. Ginebra and colleagues summarized reports of a variety of therapeutic agents with a broad spectrum of molecular weight including ions, small molecule drugs, antibiotics and growth factors that have been tested in CPCs. After being incorporated into CPCs, these molecules did not lose their therapeutic effects yet exhibited a significant burst release causing local drug concentration to exceed toxic limits [2]. Thus, it became a great challenge in the development of CPC-based drug delivery systems. Carbon nanotubes (CNTs) are cylindrical molecules formed purely by carbon atoms. This nanomaterial has emerged as a promising vehicle to transport therapeutic agents due to its hollow structure and accessible to surface functionalization to increase affinity to bioactive molecules [4,5]. Multi-walled CNTs (MWCNTs) are more amenable to surface modification compared to single-walled CNTs [6]. When carboxylic acid-functionalized MWCNTs were added into CPCs, the cement paste possessed shortened setting time while remaining biocompatible to the osteoblast cell line MC3T3-E1 [7]. When implanted subcutaneously in mice, MWCNTs did not elicit severe inflammatory responses or notable cytotoxicity and showed the same degree of healing compared to the sham operation [8]. These results led us to believe that incorporation of CNTs can be a promising approach to manipulate drug release profile of CPCs.

Here we demonstrated the drug delivery activity of a MWCNT-reinforced monetite (DCPA, $CaHPO_4$)-based cement. Z-Leu-Leu-Leu-al (MG132) was introduced as the model drug. This proteasome inhibitor effectively inhibits NF-κB-mediated gene expression, which plays the central role in osteoclast-mediated bone resorption and destruction

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[9,10]. Both the CPC and MWCNT-reinforced CPC were able to carry MG132 which remained effective in inhibiting cytokine-induced NF-κB activation; however differences in release pattern and in cytotoxicity were observed. Our data indicated that incorporating MWCNTs in the CPC may attenuate the kinetics of burst release suggesting a possible solution to optimize the drug delivery property of CPCs.

2. Materials and methods

2.1. Drug-loaded CPC preparation

All reagents for the CPC synthesis were purchased from Fisher Scientific (Fair Lawn, NJ, USA) if not specifically noted. The DCPA composite was produced by mixing 1.9 g $Ca(OH)_2$, 1.2 g $NaHCO_3$, and 2.6 ml 85% H_3PO_4 in 1.9 ml deionized (DI) water followed by 1 min of 1200 W microwave to generate dry material, which was then grinded into fine powder [11]. The cement compositions for the experiment were prepared by homogeneously vortexing 1 g DCPA powder in 0.5 ml DI water with or without 0.01 g carboxylic acid-functionalized MWCNTs (Sun Innovation, CA, USA) and with 50 μl of 10 mM MG132 (EMD Millipore, Germany) in dimethyl sulfoxide (DMSO) or 50 μl DMSO alone. The CPC paste was left to cure in a 6-well plate at 37 °C followed by ethanol wash and UV sterilization. Each pellet of CPC/DMSO, MG132-loaded CPC (CPC/MG132), CNT-reinforced CPC (CPC/MWCNT/DMSO) or MG132-loaded CNT-reinforced CPC (CPC/MWCNT/MG132) was immersed in 2 ml medium used to culture indicator cells, and this conditioned medium (CM) was collected at the time point as indicated and assayed as described below. The morphological features of the cements were visualized by scanning electron microscopy (SEM, S-4800, Hitachi).

2.2. Cytotoxicity assays

Murine osteoblastic MC3T3-E1 cells (ATCC, VA, USA) were seeded in a 12-well plate and cultured in minimum essential medium Eagle, alpha modification (α-MEM, HyClone, UT, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 24-hour prior to the experiment. Culture medium was then substituted by the CM, which was harvested 24 h after incubating with the CPC or MWCNT/CPC loaded with or without MG132. After 24 h, the culture was washed with phosphate-buffered saline (PBS) and treated with 1 mg/mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, MO, USA) for 4 h. The formazan precipitate was dissolved in DMSO and detected by a microplate reader at a wavelength of 570 nm to assess live cells.

2.3. Luciferase reporter assays

Human embryonic kidney 293 cells (ATCC, VA, USA) were seeded in a 12-well plate and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, UT, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 24-hour prior to the experiment. The reporter plasmid 3XκB-Luc, a generous gift from Dr. Brian Ashburner (University of Toledo, OH, USA) contained 3 copies of the NF-κB binding site from the major histocompatibility complex class I gene upstream of the luciferase reporter gene. Cells were transfected with reporter construct (0.2 μg) and β-galactosidase-expressing plasmid (0.2 μg). After 24 h, culture medium was substituted by the CM collected at different time points (Fig. 1), and the cell culture was further incubated for another hour followed by an 8-hour treatment of TNFα (20 ng/ml, Invitrogen, CA, USA). Cell lysates were prepared for luciferase activity measurement. β-Galactosidase activity was also measured as internal control.

2.4. Quantitative real-time PCR

Murine pre-osteoclastic RAW264.7 cells (ATCC, VA, USA) were seeded in a 6-well plate and cultured in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 24-hour prior to the experiment. Culture medium was then substituted by the CM harvested 24 h after incubating with the cement sample. In addition, the cell culture was treated with TNFα (50 ng/ml) or left untreated. Cellular RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) after 5 days and reversely transcribed to generate cDNA by M-MLV reverse transcriptase (Promega, WI, USA). Amplification of the cDNA sample was carried out in a mixture of a primer pair targeting cathepsin K, an indicator of osteoclast upregulation, and SsoFast EvaGreen Supermix (Bio-Rad, CA, USA) for 40 cycles of 2-step process (30 s at 95 °C and 60 s at 62 °C) performed on an Eppendorf Realplex thermal cycler. The CT value was obtained and normalized based on GAPDH reading.

3. Results and discussion

NF-κB manipulates cell behaviors by activating expression of various proteins, for example in promoting cell survival or regulating cell differentiation [12,13]. Activation of NF-κB itself involves disruption of the inhibitor of NF-κB (IκB) via proteasome-mediated degradation where proteasome inhibitors target this machinery to block NF-κB-directed signal transduction [14–16]. RANKL and TNFα are two cytokines known to stimulate NF-κB activation leading to osteoclast differentiation and secretion of hydrolytic enzymes, such as cathepsins and matrix metalloproteases (MMPs) [17–20]. As a potent proteasome inhibitor, MG132 can potentially be used to attenuate osteoclastic resorption. However, since MG132 is also known to induce apoptosis, burst release of this drug can be harmful to the surrounding tissue [15,21]. Thus, we first investigated the potential toxic effect of the drug/cement complex.

MG132 was loaded into the CPC pellet by adding MG132/DMSO during cement preparation. One gram of CPC contained 237.8 μg MG132. The addition of the drug did not result in the change of crystalline structure of the cement (Fig. 2a–d).

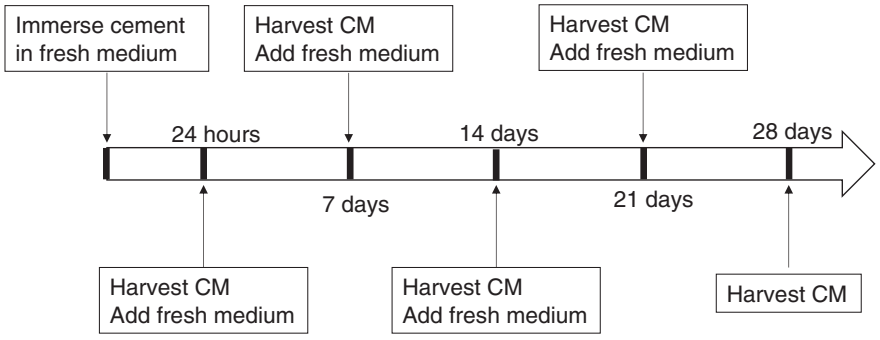

Fig. 1. The CM incubated with CPC or CPC/MWCNT with or without loaded MG132 was harvested at the indicated time point.