Biomaterials 34 (2013) 6147–6156

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

N-acetyl cysteine as an osteogenesis-enhancing molecule for bone regeneration

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ARTICLE INFO

Article history:
Received 15 April 2013
Accepted 30 April 2013
Available online 24 May 2013

Keywords:
Anti-oxidant
Chemical biology
Growth factor
Multi-function
Osteostimulation

ABSTRACT

Bone regeneration often requires cues from osteogenesis-inducing factors for successful outcome. N-acetyl cysteine (NAC), an anti-oxidant small molecule, possibly modulates osteoblastic differentiation. This study investigated the potential of NAC as an osteogenesis-enhancing molecule *in vitro* and *in vivo*. Various concentrations of NAC (0, 2.5, 5.0, and 10 mM) were added to rat bone marrow stromal cell or osteoblastic cell culture in media with or without dexamethasone. The results showed marked enhancement of alkaline phosphatase activity and mineralized matrix formation together with consistent upregulation of bone-related gene markers such as collagen I, osteopontin, and osteocalcin in the osteoblastic culture with addition of 2.5 or 5.0 mM NAC regardless of the presence of dexamethasone. Micro-CT-based analysis and histological observation revealed that addition of NAC to a collagenous sponge implanted in a critical size cortical bone defect (3.0 mm × 5.0 mm) in rat femur yielded acceleration and completion of defect closure, with thick, compact, and contiguous bone after 6 weeks of healing. In contrast, with sponge alone, only sparse and incomplete bone regeneration was observed during the matching healing period. These results indicate that NAC can function as an osteogenesis-enhancing molecule to accelerate bone regeneration by activating differentiation of osteogenic lineages.

1. Introduction

Massive bone defects referred to as critical size defects, due to trauma, tumor resection, revision arthroplasty, or severe periodontitis cannot heal spontaneously and require some form of biological augmentation for reconstruction. Development of osteogenesis-inducing factors has been required for restoration and enhancement of bone regeneration capabilities. Recently, chemical approaches have attracted interest in stem cell and regeneration biology. Several small molecules have been shown to modulate the fate and self-renewal of stem cells and the function of precursor and terminally differentiated cells [1–3]. The small molecule approach generally offers distinct advantages over traditional recombinant peptide-based or genetic approaches in cellular pharmacokinetics. Small molecules typically provide a high degree of temporal control over protein function, inducing rapid inhibition or activation [1,4,5], and, the effects of such molecules can be sensitively regulated in a concentration-dependent manner [6–8]. Furthermore, it may be possible to generate a desired phenotype in a synergistically favorable manner with a single molecule by simultaneously modulating multiple specific targets within a protein family or across different protein families or types of cell [9].

N-acetyl cysteine (NAC) is an anti-oxidant amino acid derivative with small molecular weight (163.19). This molecule is soluble in water, but can rapidly enter the cytoplasm by means of organic anion transporters in the cell membrane. N-acetyl cysteine has anti-oxidant capabilities, allowing it to function both intra- and extracellularly as a thiol compound. In earlier studies, we demonstrated how these capabilities allowed NAC to assist in tissue regeneration on biomaterial, preventing wound infection and

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http://dx.doi.org/10.1016/j.biomaterials.2013.04.064

enhancing the cytocompatibility of the material. Loss of cell viability and deterioration of cellular function in gingival fibroblasts and bone marrow-derived osteoblasts due to representative wound infection pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes* were prevented on an NAC-containing scaffold, possibly due to its anti-oxidant and bacteriostatic properties [10]. In addition, we demonstrated that NAC permitted various tissue-forming cells such as gingival fibroblasts, dental pulp cells, and osteoblasts to survive and function on dental and orthopedic biomaterials such as polymethyl methacrylate (PMMA)-based orthopedic cement and dental resin, resin-modified glass ionomer, mineral trioxide aggregate, and organic and inorganic bone substitute, both *in vitro* and *in vivo* [11–22]. These findings indicate that NAC diversely functionalizes biomaterials, thus assisting in tissue regeneration.

Some recent reports have also highlighted the capability of NAC to regulate cellular differentiation in various types of cell from both hard and soft tissues. N-acetyl cysteine induced dose-dependent inhibition of proliferation without toxicity or apoptosis, upregulation of differentiation-related genes, and expression of cell-specific differentiated structures and functions in human primary epithelial and cancer cells [23,24]. Rat and human dental pulp stromal cells were protected by NAC from resin monomer-induced cell death, possibly by increasing cellular differentiation [25]. One mouse calvarial cell culture study suggested that NAC had the potential to stimulate osteoblastic differentiation [26]. Our previous study demonstrated that incorporation of NAC into PMMA-based bone cement resulted in consistent upregulation of bone-related gene expression in cultured osteoblasts to levels beyond not only that on untreated bone cement, but also that on polystyrene under non-toxic culture conditions [18]. These findings also suggest that NAC functions as an osteogenesis-enhancing molecule, activating osteoblastic differentiation in addition to its anti-oxidant and detox functions. Moreover, if delivered into massive bony defect, it might enhance bone regeneration and promote bone defect healing. This would pave the way to the development of a new type of multi-functionalized 'smart' bone biomaterial with upgraded cytocompatibility, anti-infection capability, and activation of osteogenic cellular function.

The objectives of this study were 1) to determine the effect of NAC on differentiation of mesenchymal stem cells and osteoblastic cells, and 2) investigate whether application of NAC promoted bone healing in a rat femoral critical size defect model. Furthermore, we sought to further clarify the mechanisms and pathways involved in NAC-mediated promotion of osteogenic differentiation.

2. Materials and methods

2.1. NAC preparation

An NAC stock solution was prepared by dissolving NAC powder (Sigma–Aldrich Japan, Tokyo, Japan) in HEPES buffer (1 mol/L stock, pH 7.2).

2.2. Rat bone marrow stromal cell culture

Bone marrow stromal cells were isolated from the femurs of 8-week-old, male, Sprague–Dawley rats. Some of the cells were grown in osteogenic media to induce differentiation (hereinafter referred to as "osteoblastic cells"). The osteogenic media consisted of alpha-modified Eagle's medium (α-MEM) supplemented with 15% fetal bovine serum, 50 μg/ml ascorbic acid, 10 mM Na-β-glycerophosphate, antibiotic-antimycotic solution, and 10^{-8} M dexamethasone [27]. For culture without induction of differentiation, some of the isolated cells were also grown in the same medium but without dexamethasone (hereinafter referred to as "bone marrow stromal cells"). All cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. When 80% confluent, the cells in both types of medium were detached using 0.25% trypsin-1 mM EDTA-4Na and seeded onto 12-well, culture-grade, polystyrene plates at a density of 3×10^4 cells/cm^2 in 1 ml osteogenic or growth medium. Immediately after seeding, 0, 2.5, 5.0, or 10.0 μl NAC stock solution was added to the culture (final concentration of 0, 2.5, 5, or 10 mM NAC per culture). Three culture conditions (the osteoblastic cell culture in the osteogenic medium or the growth medium, or the bone marrow stromal cell culture in the growth medium) were employed to evaluate the effect of NAC on differentiation of mesenchymal stem cells into osteoblastic lineages and the function of osteoblastic cells. Each culture medium with its respective concentration of NAC was renewed every 3 days. This study protocol was approved by the University of California at Los Angeles Chancellor's Animal Research Committee.

2.3. Alkaline phosphatase activity

Alkaline phosphatase activity (ALP) of the culture was examined at day 5 using image-based and colorimetry-based assays. For the image analysis, cultured cells were washed twice with Hanks' solution, and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphthol AS-MX phosphate and 1.8 mM fast red TR for 30 min at 37 °C. The ALP-positive area on the stained images was calculated as [(stained area/total dish area) × 100](%) using an image analyzer (ImageJ, NIH, Bethesda, ML). For colorimetry, cells were rinsed with PBS and incubated at 37 °C for 15 min in the presence of p-nitrophenylphosphate (250 ml) (LabAssay ALP, Wako Pure Chemicals, Osaka, Japan). ALP activity was evaluated as the amount of nitrophenol released by the enzymatic reaction and measured using an ELISA reader at 405 nm. Each value was standardized by the number of cells in each duplicate culture, counted with a hematocytometer.

2.4. Mineralization assay

Mineralization capability of the culture at day 14 was examined by image-based and colorimetry-based assays. First, von Kossa stain was utilized to visualize the mineralized area of the culture. The cells were fixed using a 50% ethanol/18% formaldehyde solution for 30 min. The cultures were then incubated with 5% silver nitrate under UV light for 30 min. Finally, the cultures were washed twice with ddH_2O and incubated with 5% sodium thiosulfate solution for 2–5 min. The mineralized nodule area defined as [(stained area/total dish area) × 100] (%) was measured using an image analyzer (ImageJ, NIH, Bethesda, ML). For the colorimetric evaluation of calcium deposition, cells were washed with PBS and incubated overnight in 1 ml of 0.5 M HCl solution with gentle shaking. The solution was mixed with o-cresolphthalein complexone in an alkaline medium (Calcium Assay Kit, Cayman Chemical Company, MI) to produce a purple cresolphthalein complexone complex. Color intensity was measured in terms of absorbance at 570 nm using an ELISA reader.

2.5. Gene expression analysis

Gene expression was analyzed using reverse transcriptase-polymerase chain reaction (RT-PCR) on culture days 3, 7 and 14. Total RNA in these cultures was extracted using TRIzol (Invitrogen, Carlsbad, CA) and a purification column (RNeasy, Qiagen, Valencia, CA). Following DNAse I treatment, reverse transcription of 0.5 μg total RNA was performed using MMLV reverse transcriptase (Clontech, Carlsbad, CA) in the presence of oligo(dT) primer (Clontech, Carlsbad, CA). Polymerase chain reaction was performed using Taq DNA polymerase (EX Taq; Takara Bio, Madison, WI) to detect bone morphogenetic protein 2, Runx2 (runt-related gene 2)/Cbfa1 (core binding factor-1), collagen I, osteopontin and osteocalcin mRNA using primer designs and PCR condition established previously [27,28]. The PCR products were visualized on 1.5% agarose gel by ethidium bromide staining. Band intensity was detected and quantified under UV light and normalized with reference to GAPDH mRNA.

2.6. Collagen production

To quantify collagen production, Sirius red staining-based colorimetric assay was employed. Cultures at day 14 were washed with pre-warmed 1 × PBS at 37 °C for 1 min and fixed with Bouin's fluid for 1 h at room temperature. The cultures were washed with ddH_2O and treated with 0.2% aqueous phosphomolybdic acid (PMA) for 1 min. Then, the cultures were washed again with ddH_2O and stained with Sirius red dye (Direct Red 81, Sigma–Aldrich) dissolved in saturated aqueous picric acid (pH 2.0) at a concentration of 100 mg/100 ml for 90 min with mild shaking. The cultures were washed with 0.01 N hydrochloric acid for 2 min to remove all non-bound dye. Afterwards, 600 μl of 0.1 N sodium hydroxide was added to dissolve the staining using a microplate shaker for 30 min at room temperature. Then, color intensity was measured in terms of absorbance at 550 nm using an ELISA reader.

2.7. Osteocalcin expression analysis

Osteocalcin (OCN) levels in the culture media were analyzed immunochemically at day 14. The osteocalcin kit (Rat Osteocalcin Assay Kit, Biomedical Technologies Inc., MA) involved a monoclonal antibody directed against the N-terminal region of OCN and a goat polyclonal secondary antibody. The color was developed using an horseradish peroxidase-conjugate of a donkey anti-goat IgG. Tetramethylbenzidine (TMB) was used as a coloring agent (Chromogen). The strength of coloring was measured by absorbance at 450 nm.