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N-acetyl Cysteine Alleviates Cytotoxicity of Bone Substitute
M. Yamada, T. Ueno, H. Minamikawa, N. Sato, F. Iwasa, N. Hori and T. Ogawa

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N-acetyl Cysteine Alleviates Cytotoxicity of Bone Substitute

ABSTRACT
Lack of cytocompatibility in bone substitutes impairs healing in surrounding bone. Adverse biological events around biomaterials may be associated with oxidative stress. We hypothesized that a clinically used inorganic bone substitute is cytotoxic to osteoblasts due to oxidative stress and that N-acetyl cysteine (NAC), an antioxidant amino acid derivative, would detoxify such material. Only 20% of rat calvaria osteoblasts were viable when cultured on commercial deproteinized bovine bone particles for 24 hr, whereas this percentage doubled on bone substitute containing NAC. Intracellular ROS levels markedly increased on and under bone substitutes, which were reduced by prior addition of NAC to materials. NAC restored suppressed alkaline phosphatase activity in the bone substitute. Proinflammatory cytokine levels from human osteoblasts on the bone substitute decreased by one-third or more with addition of NAC. NAC alleviated cytotoxicity of the bone substitute to osteoblastic viability and function, implying enhanced bone regeneration around NAC-treated inorganic biomaterials.

KEY WORDS: apoptosis, reactive oxygen species (ROS), anti-oxidant, bone regeneration, inorganic biomaterial.

INTRODUCTION
An inorganic bone substitute as represented by deproteinized bovine bone particles is frequently used in preprosthetic alveolar ridge augmentation. Bone substitute is placed directly within the bone cavity or on the surface of a decorticated osseous ridge to serve as a three-dimensional solid scaffold for osteogenic cell-induced bone formation (Somnathan and Simunek, 2006). Osteocompatibility of the bone substitute is crucial to the success of bone augmentation, and an adverse osteoblastic response may result in impairment of bone formation and prolongation of healing time. Although there are concerns regarding the cytocompatibility of inorganic materials (Ignjatovic et al., 2006; Liu et al., 2008), the compatibility of commercially available inorganic bone substitutes with osteoblasts remains to be clarified.

Recently, certain types of artificial devices/materials have been reported to exert an adverse biological impact on cells. Expanded polytetrafluoroethylene (e-PTFE) induced neutrophil death (Kaplan et al., 1994; Nadzam et al., 2000; Patel et al., 2007), and reduced mitochondrial activity in both periodontal ligament fibroblasts and in an osteosarcoma cell line (Alpar et al., 2000) by contact stimuli. Titanium alloy activated monocytes and macrophages, resulting in damage to co-cultured osteoblasts (Tsaryk et al., 2007). Octacalcium phosphate induced nitric oxide production in cultured articular chondrocytes, possibly leading to degenerative arthropathies (Carter et al., 2002). Inflammatory cytokine production in osteoblasts was increased in the presence of hydroxyapatite particles (Lenz et al., 2009). Although the underlying pathological mechanism remains to be elucidated, an association has been suggested between the adverse biological effects induced by biomaterials and excessive generation of intracellular reactive oxygen species (ROS), which results in oxidative stress on cells (Luo et al., 1998; Tsaryk et al., 2007; Keegan et al., 2008).

N-acetyl cysteine (NAC) is an antioxidant amino acid derivative, and a sulphydryl group, the functional moiety of NAC, directly neutralizes ROS (Schweikl et al., 2006; Spagnuolo et al., 2006). NAC can be incorporated into a cell and deacetylated into L-cysteine, a precursor of glutathione (Zafarullah et al., 2003; Schweikl et al., 2006), which plays a central role in intracellular redox balance (Taylor et al., 2002). This antioxidant capacity can protect cells from oxidative stress by directly scavenging extracellular ROS and compensating for the depletion of intracellular glutathione levels (Gillissen et al., 1997; Gillissen and Nowak, 1998). In fact, NAC prevented the suppression of cell viability and function in fibroblasts and dental pulp cells caused by resin (Att et al., 2009; Yamada et al., 2009), which exhibits cytotoxicity via oxidative stress on cells (Kojima et al., 2008). We hypothesized that NAC would
detoxify inorganic bone material if such material is cytotoxic to osteoblasts due to oxidative stress. The objectives of this study were to (1) determine whether commercial inorganic bone substitute negatively affects the viability, behavior, and function of osteoblasts in association with oxidative stress, and, if so, (2) explore the mechanisms underlying that cytotoxicity, and (3) determine whether NAC detoxifies that material and prevents osteoblast death and dysfunction.

**MATERIALS & METHODS**

**Bone Substitute and NAC Preparation**

A 0.04-g quantity of deproteinized bovine cancellous bone particles with a diameter of 0.25-1.0 mm (Bio-oss® Cancellous, Osteohealth, Shirley, NY, USA) was placed in each well on a culture-grade polystyrene 12-well plate. We prepared a NAC stock solution by dissolving NAC powder (Sigma-Aldrich, St. Louis, MO, USA) in HEPES buffer (1 mol/L stock, pH 7.2) which had been shown to not influence osteoblast viability (Yamada and Ogawa, 2009). We prepared the NAC-treatment solution by mixing the NAC stock solution with alpha-modified Eagle’s medium (α-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD, USA) to a volume ratio of 1:49. Immediately before cell seeding, a 250-μL quantity of α-MEM or NAC-treatment solution was added to the bone substitute.

**Rat Osteoblastic Culture**

Calvarial osteoblasts isolated from 8-week-old male Sprague-Dawley rats were grown with osteoblastic media as described in the Appendix. The cells were seeded onto polystyrene, untreated bone substitute, or NAC-treated bone substitute at a density of 3 x 10⁴ cells/cm² in 1.0 mL osteoblastic media without ascorbic acid. A polystyrene culture containing 5 mM NAC was also prepared for evaluation of NAC cytocompatibility. The medium was renewed every 3 days.

To evaluate the biological effect of bone substitute on osteoblasts under non-physical contact conditions, we used a non-contact co-incubation model using a culture insert chamber with a transwell bottom (0.04-μm pore filter) (Millicell, Millipore, Bedford, MA, USA). The chamber, containing 0.04 g bone substitute and 250 μL α-MEM or NAC-treatment solution, was suspended above a polystyrene substrate on which rat osteoblasts were seeded to a density of 3 x 10⁴ cells/cm² in 1.0 mL osteoblastic media without ascorbic acid. The bottom of the chamber was submerged at a distance of 3.0 mm from the culture. These study protocols were approved by the University of California Los Angeles Chancellor’s Animal Research Committee.

**Cell Viability, Intracellular ROS, Glutathione Level, and Osteoblastic Function Analysis**

Annexin-V-based flow cytometric cell viability and apoptosis evaluation, attached cell count, intracellular ROS quantification, and confocal laser microscopic analysis of cell morphology and intracellular ROS localization were performed in rat osteoblast cell culture on polystyrene, bone substitute, and on polystyrene under bone substitute at 24 hrs after seeding. Osteoblast functional activity on polystyrene and on bone substitute was assessed with alkaline phosphatase (ALP) staining at day 7. Total intracellular glutathione level was quantified in osteoblast polystyrene culture added with or without NAC at 24 hrs after seeding. Methodologies of all assays are described in the Appendix.

**Evaluation of Inflammatory Responses of Osteoblasts on Bone Substitute**

Inflammatory cytokine [interleukin (IL)-1β, IL-2, IL-4, IL-6, and IL-8] production of human bone-marrow-derived mesenchymal stem cells on polystyrene, the untreated, or the NAC-added bone substitute at day 2 was assessed by means of the multiplex cytokine array system. Methodological details are described in the Appendix.

**Statistical Analysis**

All experiments were repeated 3 times, except for the cell morphology experiment in which 6 samples were evaluated. All repeated measurement results were expressed as means ± SD, and significant differences (p < 0.05) among the experimental groups were evaluated with a one-way ANOVA or Student’s t test. When appropriate, the Student-Newman-Keuls multiple comparisons test (SNK test) was used post hoc.

**RESULTS**

**NAC Increases Viability of Osteoblasts on Bone Substitute**

Flow cytometric analysis revealed that only 20% of osteoblasts survived on deproteinized bovine bone, in contrast to 90% on polystyrene, at 24 hrs after seeding (p < 0.01, SNK test) (Fig. 1A and Appendix Fig. 1). Cell death on bone substitute was characterized by apoptosis. Suppressed osteoblast viability on the substitute was ameliorated by pre-treatment with NAC. Percentage of viable cells on the material increased two-fold by the pre-addition of NAC (p < 0.01). NAC reduced the percentage of early apoptotic cells on the bone substitute from 45% to 26%. Addition of NAC showed no effect on viability or apoptotic appearance of osteoblasts on polystyrene (p > 0.05).

**NAC Ameliorates Impaired Osteoblast Adhesion and Marked Intracellular ROS Generation on Bone Substitute and Increases Cellular Glutathione Levels**

Confocal laser microscopy revealed largely expanded osteoblasts developing a cytoskeleton and cellular processes with little ROS detection on polystyrene at 24 hrs after seeding, whereas attached osteoblasts on bone substitute were round, small, and filled with intracellular ROS (Appendix Fig. 2). Even more largely expanded osteoblasts and lower ROS production were observed on NAC-treated bone substitute than on untreated material. Area, perimeter, and Feret’s diameter of osteoblasts on
untreated bovine mineral were only 15% or less of those on polystyrene (p < 0.01) (Fig. 1B). However, pre-addition of NAC yielded a greater than three-fold increase in values for those parameters compared with those for untreated material. The number of attached cells on bone substitute was less than one-tenth of the number on polystyrene at 24 hrs after seeding (p < 0.01) (Fig. 1C). NAC increased attached cell number on bone substitute by 160% compared with that on untreated substitutes (p < 0.01). Intracellular ROS levels in osteoblasts exhibited a 16-fold increase on untreated bovine mineral compared with that on polystyrene (p < 0.01) (Fig. 1D), and decreased to less than half of that on untreated materials on NAC-added substitute (p < 0.01). Numbers of attached cells, cell morphology, and intracellular ROS levels on polystyrene at 24 hrs after seeding were not affected by NAC (Figs. 1B-1D). NAC-addition resulted in a 70% increase in cellular glutathione levels of osteoblastic polystyrene culture at 24 hrs after seeding (p < 0.01, Student’s t test) (Fig. 1E).

**Figure 1.** Cell viability and apoptosis analysis by Annexin V-based flow cytometry in osteoblasts cultured on polystyrene with or without NAC and on deproteinized bovine bone particles with or without NAC at 24 hrs after seeding (A). Percentages of viable cells (quadrant 3 in upper images), early apoptotic cells (quadrant 4), and late-apoptotic cells (quadrant 2) are shown. Results of cell morphometric measurement (B), attached cell number quantified by Calcein fluorophotometry (C), and intracellular ROS level evaluated by DCF-DA fluorophotometry (D) in cultures on polystyrene or on bone substitute with or without NAC at 24 hrs after seeding. Poly, polystyrene culture; BS, culture on bone substitute with or without NAC; NAC, polystyrene culture with NAC. Total glutathione quantification in osteoblastic polystyrene culture added with or without 5.0 mmol/L NAC at 24 hrs after seeding (E). UT, polystyrene culture without NAC; NAC, polystyrene culture with 5.0 mmol/L NAC. Data are mean ± SD [N = 3 (A, C, D, E), N = 6 (B)]. *p < 0.01 [SNK-test].

**NAC Restores Suppressed ALP Activity on Bone Substitute**

On day 7, ALP activity was hardly detected in cells on bovine mineral, in contrast to extended and intensive positive reaction for ALP staining on polystyrene (Fig. 2 and Appendix Fig. 3). The percentage of ALP-positive area was 0.05% on bone substitute, whereas it was 80% on polystyrene. However, ALP activity on bone substitute was substantially elevated by the pre-addition of NAC. The percentage of the ALP-positive area on bone substitute increased up to 50% by NAC pre-treatment (p < 0.01).

**NAC Reduces Pro-inflammatory Cytokine Production in Osteoblasts on Bone Substitute**

Cytokine multiplex analysis revealed that human osteoblasts on bone substitute secreted a 4- to 12-times greater amount of pro-inflammatory cytokines such as IL-1β, IL-2, IL-4, IL-6, and IL-8 than those on polystyrene at day 2 (p < 0.01) (Fig. 3). Pre-addition of NAC to bone substitute reduced cytokine production in human osteoblasts on bone substitute by 40-60% (p < 0.01).

**Harmful Effect of Bone Substitute on Osteoblasts under Non-contact Conditions Alleviated by NAC**

Ninety-six percent of osteoblasts cultured on polystyrene were viable at 24 hrs after seeding. This percentage decreased to 88% by non-contact co-incubation with bone substitute (p < 0.01).
Glutathione and disturbs the cellular redox balance, resulting in oxidative stress (Feinendegen, 2002). Stressed cells undergo oxidative challenge such as lipid peroxidation and DNA strand breaks, with dysregulation of cellular survival or differentiation-related signaling pathways (Feinendegen, 2002; Schweikl et al., 2006), leading to induction of apoptosis and cellular dysfunction (Yamada et al., 2008; Att et al., 2009; Tsukimura et al., 2009).

Bone substitute has the inherent potential to induce oxidative stress. Generally, organ-derived bone substitutes undergo some type of physicochemical treatment during manufacturing, including deproteination and inactivation of pathogens (Tadic and Epple, 2004), which results in alteration of surface properties and the addition of chemical remnants to the material (Carter et al., 2002; Bertazzo and Bertran, 2008). This implies that xenogenetic bone replacement material may biologically influence cells not only by contact stimuli, but also by release of certain chemical agents accompanying the material’s surface dissolution (Accorsi-Mendonca et al., 2008). In this study, osteoblast adverse responses were seen when osteoblasts were directly seeded onto the deproteinized bovine bone substitute and on underlying polystyrene under non-contact conditions. It will be of great interest for future research to identify oxidative-stress-inducing factors in inorganic bone substitute, which are suspected to include the negative influence of surface properties on cell adhesion (Dent et al., 2003), the elution of chemical substances, and ROS generation (Stefaniak et al., 2009) originating in the dissolution of materials.

N-acetyl cysteine reduced the adverse biological effects of bone substitute on osteoblasts in the present study. Its ability to detoxify oxidative-stress-evoking toxic material has been well-proven. Suppressed viability and function in extracellular matrix-producing cells on polymethyl methacrylate (PMMA)-based resin were recovered by the addition of NAC to the resin (Att et al., 2009; Yamada et al., 2009). Mechanisms underlying the detoxification of biomaterial by NAC are thought to be wide-ranging. NAC can directly scavenge ROS and toxic compounds with its functional moiety (Schweikl et al., 2006; Yamada and Ogawa, 2009). In addition, NAC has the cellular pharmacodynamics to elevate intracellular glutathione levels, as shown in the present study, which indicated that NAC may enhance the anti-oxidant resistance of osteoblasts against oxidative stress from materials (Tsukimura et al., 2009). Furthermore, NAC might modulate cysteine-containing transcriptional factors such as nuclear factor kappa B, which regulates cell survival and differentiation (Paranjpe et al., 2007).

A variety of immune and tissue-forming cells produces pro-inflammatory cytokines. Some type of biomaterial may increase osteoblastic pro-inflammatory cytokine production, especially IL-6 and IL-8 (Huang et al., 2005; Lenz et al., 2009). IL-6 acts as a multiple major mediator of pro-inflammatory processes such as B-cell differentiation (Ishimi et al., 1990). IL-8 is a chemokine to induce neutrophil chemotaxis and activation (Baggiolini et al., 1994). Dysregulated generation of these cytokines in bone tissue may lead to local inflammatory development, increased osteoclastic bone resorption, and reduced bone formation (Vermes et al., 2001). Moreover, these pro-inflammatory cytokines are involved in cross-talk between osteoblasts and immune cells.
Detoxified Bone Substitute by NAC (Rauner et al., 2007). It will be of great interest for further research to examine the influences of bone substitute and the effect of NAC on the osteoimmunological network.

Biological amelioration of bone substitute may lead to the upgrading of surrounding bone regeneration, i.e., acceleration of bone formation and enhancement of bone volume and bone-material integration strength. Indeed, NAC detoxified PMMA-based bone cement, resulting in remarkably enhanced bone-contact area, surrounding bone volume, and biomechanical strength at the bone-material interface as compared with the original cement alone (Tsukimura et al., 2009). NAC-induced improvement in the biocompatibility of bone substitute may functionalize the material and offers potential in the development of new breakthrough bone substitutes.

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REFERENCES


