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ScienceDirect
Acta Biomaterialia 5 (2009) 2963–2973
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Chemodynamics underlying *N*-acetyl cysteine-mediated bone cement monomer detoxification

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Received 10 February 2009; received in revised form 20 April 2009; accepted 23 April 2009
Available online 3 May 2009

Abstract

Methyl methacrylate (MMA)-based bone cement monomer is cytotoxic. *N*-Acetyl cysteine (NAC), a cysteine derivative, may alleviate this toxicity by inactivating the monomer components with its sulfhydryl moiety. This study examined the chemical interaction dynamics between bone cement monomer and NAC resulting in detoxification of the monomer. A monomer/NAC mixture was prepared by mixing and incubating a commercially available MMA-based bone cement monomer with NAC for various time periods of 1 min, 1 h, 6 h and 24 h. Rat bone marrow-derived osteoblastic cells were cultured with either the monomer/NAC mixture or the monomer alone. Only 17% of the cells were viable 24 h after seeding in the culture containing the monomer alone. The proliferation rate and alkaline phosphatase activity of the cells were substantially reduced under this condition. In contrast, when cultured with the monomer/NAC mixture, the viability and function of the cells were improved with increasing time of monomer/NAC incubation. For instance, the monomer/NAC mixture that was pre-reacted for 1 min increased cell viability from 17% to 55%. The monomer/NAC mixture that was pre-reacted for 24 h nearly completely restored cell viability, proliferation and ALP activity to the level of an untreated control culture. The DPPH radical-scavenging capacity of monomer/NAC mixture decreased with an increase in their reaction time, indicating time-dependent depletion of the NAC anti-oxidant moiety. Within the limit of this experimental condition, these data demonstrate the immediate initiation and rapid completion of bone cement monomer/NAC interaction, resulting in abrogation of the monomer's cytotoxicity.

Keywords: Apoptosis; Reactive oxygen species (ROS); Cytotoxicity; Anti-oxidant; PMMA resin

1. Introduction

Polymethyl methacrylate (PMMA)-based bone cement, which is prepared by mixing PMMA powder with methyl methacrylate (MMA) monomer liquid, is frequently used in orthopedic procedures such as the internal fixation of total joint prostheses and the bone reconstruction after tumor resection or skeletal fracture [1,2]. However, adverse events associated with clinical use of the cement are a serious concern. Necrosis of the surrounding bone tissue often occurs and results in failure of joint replacement [3]. Moreover, patients undergoing cement implantation occasionally develop serious systemic symptoms, such as hypotension, hypoxemia, arrhythmia, cardiac arrest, any combination of these or, at worst, unexpected death, the so-called bone cement implantation syndrome [4].

The cytotoxic effects of acrylic bone cement have been demonstrated extensively in previous experimental studies. Bone cement induced apoptosis in osteoblastic and leukocytic cell lines [5–7]. The cell cycle of osteoblast-like cells was arrested after exposure to bone cement extracts [8]. Acrylic bone cement extracts promoted inflammatory cytokine production or the expression of an osteolysis-related gene in osteoblast-like cells [9,10]. Fibrosis or necrosis was found locally in bone tissue around implanted bone

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doi:10.1016/j.actbio.2009.04.027

cement [11,12]. Residual or eluted MMA monomer is released from the cement copolymer during and after polymerization, and is regarded as one of the major causes of these adverse biological effects [13,14]. The cytotoxicity of the monomer may be associated with direct destruction of cell structure [13,15] or oxidative stress resulting from excessive intracellular generation of reactive oxygen species (ROS) caused by chemical stimuli in the resin [13,16].

N-Acetyl cysteine (NAC) is an anti-oxidant cysteine derivative and can be incorporated into cells and deacetylated into L-cysteine, which is a precursor of glutathione. Glutathione serves as the principal molecule in the intracellular anti-oxidant system [13,17,18]. Accordingly, NAC not only acts as a direct oxidant scavenger [19] but also improves intracellular glutathione systems compromised by oxidative stress [19,20]. Recently, incorporation of NAC into PMMA-based dental resin has been shown to restore the suppressed viability and function of dental pulp cells or oral fibroblasts on the resin substrate to a biologically significant degree [21–23]. Moreover, NAC has been demonstrated to detoxify PMMA-based bone cement and consequently to add osteoconductivity to the material [24].

There are several possibilities for the explanation of NAC's detoxification pathways, such as the scavenging of ROS originating from within polymerizing resin materials and improvement of intracellular redox systems in cells suffering from oxidative stress [24]. However, the mechanisms by which NAC detoxifies resin materials are not fully understood. Because the resilient (unreacted or excessive) monomer is an inherent and profound problem in bone cement materials and NAC has the potential to counteract this problem, the following specific questions have been raised: (i) Is there a direct chemical interaction between NAC and MMA monomer? (ii) Would such an interaction result in an improvement in the monomer's biocompatibility? (iii) What is the critical time point or time course of the monomer/NAC interaction, leading to alleviation of the monomer cytotoxicity? (iv) Can the monomer be detoxified completely by NAC? To address these questions, we examined the cytotoxic effects of a mixture of PMMA-based bone cement monomer with NAC that was pre-reacted for various time periods.

2. Materials and methods

2.1. Preparation of bone cement monomer and NAC

The MMA-based bone cement monomer liquid supplied in the commercially available PMMA bone cement kit (Endurance MV, DePuy Orthopaedics, Warsaw, IN) was used in this experiment. The manufacturer indicated the following monomer compositions: 98.0 wt.% MMA, 2.0 wt.% *N*,*N*-dimethyl-*p*-toluidine and 0.0075 wt.% hydroquinone. NAC (Sigma–Aldrich, St. Louis, MO) was prepared as a 1 mol l^{-1} stock solution in HEPES buffer wit the pH adjusted to 7.2. A monomer/NAC mixture was prepared by mixing and incubating the monomer with NAC (monomer:NAC solution, 4:1 by volume) in a light-resistant glass container for 1 min, 1 h, 6 h and 24 h. To evaluate the influence of HEPES buffer, the monomer/HEPES mixture was incubated for 1 min and 24 h. All premixtures were prepared immediately before they were used in experiments.

2.2. Osteoblastic cell culture

Rat bone marrow cells were prepared according to the method of Maniatopoulos et al. [25] with some modifications. Femurs were aseptically removed from 8-week-old male Sprague–Dawley rats, and the attached muscles and ligaments were removed. After washing with phosphate-buffered saline (PBS) and removal of metaphyses from both ends, the bone marrow cavity was flushed out with osteoblastic medium consisting of alpha-modified Eagle's medium supplemented with 15% fetal bovine serum, 50 μg ml^{-1} ascorbic acid, 10^{-8} M dexamethasone, 10 mM Na-β-glycerophosphate and antibiotic–antimycotic solution containing 10,000 U ml^{-1} penicillin G sodium, 10,000 mg ml^{-1} streptomycin sulfate and 25 mg ml^{-1} amphotericin B. Cells were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. At 80% confluency, the cells were detached using 0.25% trypsin–1 mM EDTA 4-Na and seeded onto 12-well culture grade polystyrene dishes at a density of 4×10^4 cells cm^{-2} in 1 ml of osteoblastic medium. Bone cement monomer and the mixture were added to the culture medium at the final concentrations of 20 and 25 μl ml^{-1}, respectively. Untreated cultures and cultures supplied with the NAC solution at a concentration of 5 μl ml^{-1} were used as experimental controls. The culture medium was not renewed throughout the entire culture period (3 days). The study protocol was approved by the University of California at Los Angeles Chancellor's Animal Research Committee.

2.3. Quantification of attached cell numbers

The number of attached cells in the culture was evaluated using Calcein-AM (Cell Counting Kit-F; Dojindo Molecular Technologies, Gaithersburg, MD), which is hydrolyzed into calcein by intracellular esterase. At day 1 of culture, the cells were gently rinsed twice with Dulbecco's PBS (D-PBS) and subsequently incubated with Calcein-AM solution in D-PBS for 30 min at 37 °C. After removing the Calcein-AM solution, the cells were gently rinsed with D-PBS again. Calcein fluorescence intensity of the culture was then measured using a fluorescence plate reader at 535 nm (excitation at 485 nm) against D-PBS as a blank.

2.4. Measurement of intracellular ROS level

The amount of intracellular ROS produced in attached cells was quantified by 5- (and 6-) carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen, Gaithersburg, MD, USA), which is absorbed intracellularly

ID	Title	Pages
1108	Chemodynamics underlying N-acetyl cysteine-mediated bone cement monomer detoxification	11