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The inhibition of infection by wound pathogens on scaffold in tissue-forming process using N-acetyl cysteine

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ABSTRACT

Prevention of local infection from wound pathogens such as Staphylococci and Streptococci is crucial for tissue regeneration. N-acetyl cysteine (NAC), an anti-oxidant amino acid derivative, has anti-microbial potential against various species. This *in vitro* study evaluated whether NAC prevented bacterial infection of gingival fibroblasts and osteoblasts on a scaffold. N-acetyl cysteine delayed growth of *Staphylococcus aureus* and *Streptococcus pyogenes* cultured in brain heart infusion (BHI) broth for 12 h in an almost dose-dependent manner (2.5, 5.0 or 10.0 mM). The number of rat gingival fibroblasts on collagen scaffolds with bacterial co-incubation was less than 30% of that in cultures without bacterial co-incubation at day 7. However, pre-addition of NAC to the scaffold yielded a number comparable with that in culture without bacteria. Fibroblasts on the scaffold with bacterial co-incubation were small, rounded and filled with bacteria and reactive oxygen species. Pre-addition of NAC, however, resulted in fibroblasts similar to those observed in culture without bacterial co-incubation. N-acetyl cysteine completely prevented devastating suppression of alkaline–phosphatase activity and extracellular matrix mineralization in osteoblastic culture on scaffolds with bacterial co-incubation. These results indicate that NAC can functionalize a scaffold with anti-infective capabilities, thus assisting healing of soft and hard tissues.

1. Introduction

Wound infection is one of the most serious issues in tissue regeneration and biomaterial implantation. Generally, certain types of Staphylococci and Streptococci such as *Staphylococcus aureus* and *Streptococcus pyogenes* are regarded as representative pathogens of wound infection [1,2]. These bacteria are Gram-positive and accrete as indigenous bacteria on body surfaces, including the skin, nose, genitals, anus and oral cavity. An acute wound arising from a cut, laceration or surgical procedure, provides an opportunity for such bacteria to enter and colonize the underlying tissue, including connective, muscle and bone tissue. Even after rigorous disinfection, the remaining bacteria can continue to grow and infiltrate the underlying tissue through gaps between the flaps of the wound until healing is complete [3,4]. Implantable medical devices and materials such as artificial skin, pacemakers, heart valves, orthopedics, dental bone-anchored prostheses and bone graft materials are also subject to the bacterial invasion scenario [3–6]. Bacterial contamination of an implantable scaffold results in tissue breakdown and degradation around the implant material.

Oxidative stress, which results from increased levels of reactive oxygen species (ROS) and a distortion of cellular redox balance, is involved in several pathological conditions. Intracellular ROS can be excessively generated due to various exogenous stimuli such as ultraviolet (UV) light, ionizing radiation, physical stimuli, various chemicals and toxins and bacterial infection [7–9], which markedly consumes the cellular anti-oxidant glutathione (GSH). Due to their high reactivity, ROS can oxidize cellular components such as lipids, proteins, and DNA and thus damage cell structure and integrity [7,10–13]. In addition, ROS induce apoptosis via a wide variety of cellular pathways, including a release of cytochrome *c* from mitochondria and lysosome membrane permeabilization [14–17]. Oxidative stress

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is strongly associated with the phagocytotic process [18–21]. Some bacterial species including *Staphylococcus aureus* and *Streptococcus pyogenes* utilize the phagocytotic process for invasion into cell [22–24]. Recent culture studies demonstrated that phagocytosis-related ROS generation caused intracellular oxidative damage leading to cell death or dysfunction in macrophages, fibroblasts and osteoblasts [25–28].

N-acetyl cysteine (NAC), an anti-oxidant amino acid derivative, is used in expectorant in respiratory medicine. This small-molecule compound (molecular weight: 163.19) can directly scavenge free radicals and toxic compounds with its functional moiety, the sulfhydryl group [7,29]. In addition, NAC is membrane-permeably incorporated into cells and rapidly metabolized into L-cysteine, a precursor of GSH, resulting in maintenance of intracellular redox balance. This strong anti-oxidant activity of NAC protects cells from oxidative stress caused by various external stimuli [7,30]. In earlier studies, we demonstrated that NAC detoxified oxidative stress-inducing biomaterials such as polymethyl methacrylate resin (PMMA) and organic and inorganic bone biomaterials, allowing maintenance of cell viability and differentiation in dental pulp cells, fibroblasts and osteoblasts in contact with those materials by reduction of oxidative stress [29,31–38]. Large quantities of direct bone deposition occurred on NAC-incorporated PMMA bone cement implanted into rat femur bone in contrast with sparse bone formation around PMMA cement alone [33]. These results indicate that the anti-oxidant properties of NAC can functionalize biomaterial, enhancing its cytocompatibility. Moreover, recent culture studies indicated that NAC also had anti-microbial potential. It was shown that NAC delayed biofilm formation of certain types of fungi and bacteria such as *Aspergillus* and *Fusarium* species [39], *Escherichia coli* [40], *Pseudomonas aeruginosa* [41], *Staphylococcus epidermidis* [42], *Staphylococcus warneri*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and other Gram-positive and -negative bacteria on polystyrene and stainless steel surfaces [43]. Other culture studies showed that NAC reduced bacterial and viral adherence on human pharyngeal or oropharyngeal epithelial cells [44,45].

The unique pharmacological properties of NAC, however, raise the following intriguing questions: 1) does NAC exert an anti-microbial effect on the representative Staphylococci and Streptococci involved in wound infection?; 2) can preparation of a tissue-engineering scaffold with NAC prevent bacterial infection and related oxidative stress in cells attaching to it?; and 3) if so, can NAC prevent loss of viability and function in both soft and hard tissue-forming cells caused by bacteria and encourage tissue formation on a scaffold, even in the presence of wound-associated pathogens? Clarification of these questions would pave the way for the development of multi-functionalized biomaterials with not only enhanced cytocompatibility, but also anti-infection capabilities. The objectives of this *in vitro* study were 1) to determine whether NAC exerted an anti-microbial effect on *S. aureus* and *S. pyogenes*; and 2) to determine whether addition of NAC to a collagen membrane or spongy scaffold protected fibroblasts or osteoblastic cells cultured on that scaffold from bacterial infection, bacterially induced oxidative stress, cell death and dysfunction under co-incubation with wound pathogens. Furthermore, we sought to further clarify the mechanisms and pathways involved in NAC-mediated anti-infective functionalization.

2. Materials and methods

2.1. NAC preparation

An NAC stock solution was prepared by dissolving NAC powder (Sigma–Aldrich, St. Louis, MO) in HEPES buffer (1 mol/L stock, pH 7.2). The solution for fibroblastic or osteoblastic culture comprised the NAC stock solution mixed with Dulbecco's Modified Eagle's medium (D-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD) or alpha-modified Eagle's medium (α-MEM, Gibco BRL Div. of Invitrogen, Gaithersburg, MD) in a volume ratio of 1–9.

2.2. Bacterial strain and culture condition

Staphylococcus aureus 209P and *S. pyogenes* GTC 262 were used in the experiment. Each bacterium was grown in 3 mL brain heart infusion broth (BHI, Becton Dickinson, Sparks, MD) in a 12-mL sterile plastic tube capped tightly and incubated at 37 °C statically overnight (16–18 h).

2.3. Effect of NAC on bacterial growth

2.3.1. Susceptibility in broth culture

Brain heart infusion broth cultures with or without NAC were used for quantitative evaluation of the effect of NAC on bacterial growth. The density of the cell cultures was adjusted photometrically so that they contained approximately 1.0×10^6 cells/mL prior to their use in the experiment. Untreated bacterial cultures and experimental cultures with addition of 2.5, 5.0 or 10.0 μl NAC (final concentration of 2.5, 5, or 10 mM per 1 mL culture medium) and bacterial culture with addition of 10.0 μl HEPES buffer were prepared for evaluation of bacterial growth in culture over 12 h incubation. Reagents were added one time at the kickoff point of incubation.

Bacterial growth was quantified by absorbance at 660 nm in the BHI broth using a microplate reader after 4, 8 and 12 h incubation. Microbial metabolic activity was also quantified by bioluminescence-based adenosine triphosphate (ATP) assay using BacTiter-Glo™ Reagent (Promega Corporation, Madison, WI). After 4, 8 and 12 h incubation, reagent was added to the bacterial broth culture and luminescent intensity measured using a microplate reader according to the manufacturer's instructions.

2.3.2. Halo test

The anti-bacterial effect of NAC was also investigated with the Halo test. Filter paper disks 6.0 mm in diameter and impregnated with 30 μl HEPES buffer or NAC solution (final concentration of 2.5, 5, or 10 mM per 30 μl) were placed on BHI agar plates on which 100 μl BHI broth containing approximately 1.0×10^8 cells/mL had been uniformly smeared in advance. After 24 h incubation at 37 °C, an image analysis was performed using ImageJ (National Institute of Health, Bethesda) to determine growth-inhibitory width, which was defined as the radius of the growth-inhibitory area (mm). Subsequently, subculture streaked with a sample from the growth-inhibitory area was used to determine viability of bacteria in that area.

2.4. Preparation of collagen scaffold

Commercial collagen membrane (Tissue Guide®, KOKEN, Tokyo, Japan) and spongy scaffold (Collaplug®, Zimmer Dental, Carlsbad, CA) made from bovine type I atelocollagen were used as the substrate for fibroblastic and osteoblastic culture. The membrane scaffold was cut into small pieces, uniform in shape and with a surface area of 44.2 mm^2 each. The spongy scaffold was trimmed into small pieces uniform in area (44.2 mm^2) and thickness (0.3 mm). Each specimen was placed on a culture-grade polystyrene 48-well plate. Immediately before cell seeding, the collagen material substrate was permeated with 50 μl NAC-treatment solution or culture basal media (D-MEM or α-MEM) alone. It was confirmed that the collagen substrate retained sufficient integrity after immersion in culture medium for 21 days to allow subjection to analysis.

2.5. Rat fibroblastic and osteoblastic culture on collagen scaffold with bacterial co-incubation

Fibroblastic cells were obtained from the palatal gingiva of 8-week-old Sprague–Dawley rats. After the animals were sacrificed, the palatal tissue was aseptically removed and washed with 1% phosphate buffered saline (MP Biomedicals, Solon, OH). The tissue was dissected into small pieces and digested with 0.25% collagenase for 12 h. The liberated cells were collected into D-MEM supplemented with 10% fetal bovine serum and an antibiotic–antimycotic solution in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. At 80% confluence, the cells were detached using 0.25% trypsin-1 mM EDTA 4-Na and seeded onto polystyrene, untreated collagen material, or NAC-treated material at a density of 4×10^4 cells/cm^2 in 0.25 mL D-MEM without the antibiotic–antimycotic supplement.

Bone marrow cells isolated from the femurs of 8-week-old male Sprague–Dawley rats were placed in osteoblastic medium consisting of α-MEM supplemented with 15% fetal bovine serum, 50 μg/mL ascorbic acid, 10^{-8} M dexamethasone, 10 mM Na-β-glycerophosphate, and antibiotic–antimycotic solution containing 10,000 units/mL penicillin G sodium, 10,000 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B. Cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. At 80% confluence, the cells were detached using 0.25% trypsin-1 mM EDTA 4-Na and seeded onto polystyrene, untreated collagen material, or NAC-treated material (Fig. 1A) at a density of 4×10^4