Effect of exogenous nitric oxide on the proliferation of a human osteoblast (HOS) cell line induced by hydroxyapatite

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ABSTRACT: Aim: The aim of this study was to test the hypothesis that the proliferation of hydroxyapatite (HA)-induced human osteoblast cell line (HOS cells) may be up-regulated by exogenous nitric oxide (NO).
Methods: HOS cells were cultured on the surface of HA with or without the presence of a NO donor, S-nitroso acetyl penicillamine (SNAP) or nitroso acetyl penicillamine (NAP). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide known as carboxy PTIO, a NO scavenger, was added in the cell cultures with or without the presence of SNAP. The cells were also pre-treated with L-N5-(1-iminoethyl)ornithine hydrochloride (L-NIO), an endothelial nitric oxide synthase (eNOS) inhibitor, or anti-integrin αV antibody before culturing on HA surfaces with or without the presence of SNAP. Medium, cells alone or cells pretreated with these inhibitors or antibodies was used as the controls. Cell proliferation was assessed by colorimetric assay.
Results: The results showed that SNAP, but not NAP, augmented HA-induced HOS cell proliferation. This modulatory effect of SNAP on HA-induced HOS cell proliferation was abolished by carboxy PTIO or anti-integrin αV antibody, but only partially reduced by L-NIO.
Conclusions: Therefore, the results of this study suggest that exogenous NO alone may up-regulate the proliferation of HOS cells attached on HA surfaces via integrin αV molecules. (Journal of Applied Biomaterials & Biomechanics 2009; 7: 29-33)

KEY WORDS: Exogenous, HOS cells, Hydroxyapatite, Integrin αV, Nitric oxide, Osteoblasts, S-nitroso acetyl penicillamine

INTRODUCTION

Hydroxyapatite (HA) is known as a ceramic material widely used for orthopedic and dental implants, since this biomaterial has the ability to stimulate osteoblast functions in vitro and in vivo with unknown mechanism(s) (1). It appears to suggest that osteoblasts initially attach on HA surfaces via osteoblast-derived integrin molecules, leading to the production of growth factors, which in turn determine cell spreading and proliferation on the surface of HA (2, 3). Nitric oxide (NO) is a gaseous molecule synthesized from metabolisms of L-arginine under nitric oxide synthase (NOS) catalyzation and it plays a crucial role in many human biological systems including bone formation (4). Three NOS isoforms, i.e. neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3), are recognized (5). The exact mechanism(s) by which NO regulates bone formation remains unclear. It seems plausible that the effect of NO on osteoblast functions may be dependent on the concentration of this gaseous molecule. Low NO concentrations may augment osteoblast proliferation (6), whereas NO concentrations higher than 1 mM may induce osteoblast apoptosis, perhaps via Bcl2 protein activation (7). Interestingly, NO seems to play a crucial role in the regulation of the host’s response to dental material (8). Our previous study indicated that exogenous NO up-regulates the production of COX2-mediated PGE2 and cyclic-AMP by a HA-induced human osteoblast (HOS) cell line (9, 10). Since exogenous NO has been shown to modulate osteoblast functions, the aim of this study was to test a hypothesis that exogenous NO at low concentrations may up-regulate HA-induced HOS cell proliferation.
MATERIALS AND METHODS

Hydroxyapatite

The HA discs (9% porosity and sintered at 1200 °C) were cut into pieces 2 × 2 × 2 mm³ in size and subsequently autoclaved. In all experiments, each HA disc was placed in the well of 96-well plates (Corning, NY, USA).

Cell culture

A human osteoblast-like cell line (HOS cells) was purchased from American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco’s modified Eagle’s medium (Sigma, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin-streptomycin (Sigma) until confluent. After harvesting and washing, a single cell suspension was prepared. NO donor, S-nitroso acetyl penicillamine (SNAP) or nitroso acetyl penicillamine (NAP) (Sigma), was dissolved in distilled water to obtain 1 mM of solution and sterilized. One million HOS cells were incubated in 1 ml of the culture medium containing 20 µM of SNAP or 20 µM of NAP (v/v). Two hundred microliters of the cell suspension containing 2 × 10⁵ cells were plated on the surface of the HA-disc in each well and incubated for 3 days at 37 °C in a humidified atmosphere and 5% CO₂ (11). Cultures containing medium only or cell suspension with or without the presence of SNAP or NAP plated in the wells were used as the controls. All cultures were in triplicate.

In the other experiments, the presence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) also known as carboxy PTIO, a NO scavenger (Sigma), was dissolved in methanol to obtain 1 mM. One million cells were incubated in 1 ml of medium containing 100 µM of carboxy PTIO (9, 10). The cells were then washed and cultured on the HA disc with or without the presence of 20 µM of SNAP. The cells with or without pre-treatment with anti-αV-integrin antibody and medium only were used as controls. All cell cultures were in triplicate and incubated for 3 days after which cell proliferation was determined as described below.

Proliferation assay

A colorimetric assay was used to determine the cell proliferation as previously described (12). Briefly, after harvesting the supernatant of 3 day cultures and gently washing with sterile phosphate buffered saline (PBS), the cells were dehydrated with 100 µl of 20% methanol for 10 min and then the solution was carefully aspirated. The cells were then exposed to 100 µl of 0.5% crystal violet for 5 min followed by extensive rinsing with PBS. The dye was released from the cells by adding 100 µl of 0.1 M Na citrate in 50% ethanol. The optical density (OD) was read at a wavelength of 540 nm using a µQuant spectrophotometer (Biotek-Instrument, Inc). The results were subtracted by the optical density of medium only.

Statistical analysis

Data was analyzed by one-way analysis of variance followed by Fischer’s least square differences using a statistical software package (SPSS, Chicago, Ill, USA).

RESULTS

Effect of NO donors

As displayed in Figure 1, the proliferation of HOS cells in the presence of SNAP or HA was significantly higher than that of HOS cells alone (p<0.05). A significantly increased cell proliferation could be observed in the HA-induced HOS cells in the presence of SNAP as compared with that of HA-induced HOS cells without the presence of SNAP (p<0.05). The presence of NAP failed to alter the proliferation of HOS cells alone and HA-induced HOS cells (p>0.05).
Effect of carboxy PTIO

Figure 2 depicts the effect of carboxy PTIO on the proliferation of HA-induced HOS cells with or without SNAP. In the presence of carboxy PTIO, the HOS cell proliferation was slightly reduced (p<0.05). The proliferation of HA-induced HOS cells was significantly increased as compared with that of HOS cells, but inhibited in the presence of carboxy PTIO (p<0.05). SNAP augmented the proliferation of HA-induced HOS cells as compared with that of HA-induced HOS cells without SNAP (p<0.05). However, the up-regulatory effect of SNAP on HA-induced HOS cell proliferation was abolished in the presence of carboxy PTIO (p<0.05).

Effect of L-NIO

As seen in Figure 3, L-NIO decreased the proliferation of HOS cells alone or HA-induced HOS cells (p<0.05). Interestingly, the proliferation of HA-induced HOS cells in the presence of SNAP and L-NIO was lower than that in the presence of SNAP, but higher than that of HA-induced HOS cells without SNAP (p<0.05).

Effect of anti-integrin αV antibody

No significant differences between the proliferation of HOS cells and anti-integrin αV-treated HOS cells could be found (p>0.05) (Fig. 4). The proliferation of HOS cells pre-treated with anti-integrin αV antibody and cultured on the HA disc was lower than that of un-treated HOS cells cultured on the HA disc (p<0.05). Furthermore, the proliferation of anti-integrin αV-treated HOS cells cultured on the HA disc in the presence of SNAP was significantly lower than that of un-treated HOS cells cultured on the HA disc (p<0.05).
The results of this study showed that the proliferation of HOS cells induced by HA was significantly increased in the presence of SNAP at low concentrations. These results are in agreement with previous reports showing that exogenous NO did increase osteoblast proliferation (6). Furthermore, carboxy PTIO is known to oxidize NO to generate nitrogen dioxide (11). Inhibition of HA-induced HOS cell proliferation by carboxy PTIO seen in this study also indicated that endogenous NO may play a crucial role during the course of HA-induced HOS cell proliferation. Fluid shear stress and estradiol-induced osteoblast functions were also dependent on endogenous NO (13, 14). Therefore, the ability of carboxy PTIO to suppress HA-induced HOS cell proliferation in the presence of SNAP was obvious.

The fact that L-NIO did inhibit HA-induced HOS cell proliferation seen in this study suggests, yet again, that NO generated by the activity of eNOS may be a prerequisite in the proliferation of HA-induced HOS cells (9, 10). Similarly, fluid shear stress and estradiol stimulated eNOS-mediated NO production by osteoblasts (13, 14). However, the results of this study showed that the deletion of endogenous NO by L-NIO did not completely inhibit the modulatory effect of SNAP on HA-induced cell proliferation, indicating that exogenous NO alone may upregulate HA-induced HOS cell proliferation. These results, therefore, support previous findings that exogenous NO has the ability to facilitate directly the osteoblast proliferation (15, 16).

The ability of anti-integrin αV antibody to abolish the effect of exogenous NO on HA-stimulated HOS cell proliferation indicates that regardless of the presence of exogenous NO, integrin αV molecules remain a key osteoblast-derived adhesion molecule to attach on HA surface as previously reported (9, 10, 17). If so, it seems plausible that exogenous NO may amplify the proliferative signals generated at the interaction site between HOS cell-derived integrin αV molecules and HA surface. This is also supported by the results of previous studies indicating that NO may intensify the signals in HA-induced HOS cells to synthesize PGE2 and cAMP.

The extrapolation of this study concerning implantation with HA in humans remains speculative. This study indicates that the proliferation of HA-induced HOS cells was determined by both endogenous and exogenous NO. Macrophages and polymorphonuclear cells in the surrounding implantation in humans have also been shown to produce NO (18, 19), indicating that cells other than osteoblasts at the implanted site may act as a cellular source of exogenous NO. NO seems to play a crucial role in maintaining the integrity of dental implants (8). One could assume, therefore, that osteoblast proliferation following HA implantation in humans may be regulated by the total amount of NO produced by different types of cells at the implanted site. If the amount of NO at the surrounding implanted materials is at physiological levels, it may modulate osteoblast proliferation. However, if the total amount of NO at the implanted site is excessive, it would induce inflammatory cell infiltration, osteoblast apoptosis and/or osteoclast activation, thereby promoting inflammatory responses and bone resorption, phenomena commonly seen in failed dental implants (20). This notion certainly needs to be investigated further.

In conclusion, this study showed that exogenous NO up-regulates HA-induced stimulated HOS cell proliferation. The effect of exogenous NO on HA-induced cell proliferation was abolished by carboxy PTIO and anti-integrin V antibody, but was only partially inhibited by L-NIO. Therefore, the results of this study suggest that exogenous NO may enhance HA-induced HOS cell proliferation in an endogenous NO-independent mechanism.

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Conflict of interest statement: None of the authors has any conflict of interest.
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