Brief Report

Effect of exogenous nitric oxide on hydroxyapatite-induced human osteoblast proliferation

Erwan Sugiatno[1,3]

Abdul Rani Samsudin[1]

Mohd. Fikrie Ibrahim[1]

Wihaskoro Sosroseno[1,2]

School of Dental Sciences[1] and Department of Immunology, School of Medical Sciences[2], Universiti Sains Malaysia, Health Campus, 16150 Kota Bharu, Malaysia; Department of Prosthondotic, Faculty of Dentistry, Gadjah Mada University, Yogyakarta 55281, Indonesia[3]

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Corresponding address:

Dr. Wihas Sosroseno

Dept. of Oral Biology, School of Dental Sciences,

Universiti Sains Malaysia, 16150 Kota Bharu,

Malaysia

Fax. 60 9 7642026

e.mail: wihaskoro@kb.usm.my

Abstract

The aim of the present study was to determine the effect of exogenous nitric oxide (NO) at low concentration on hydroxyapatite (HA)-stimulated human osteoblast proliferation. Human osteoblast-like cells (HOS cell line) were cultured on the surface of HA. Medium or cells alone was used the controls. S-nitroso acetyl penicillamine (SNAP), a NO donor, and nitroso acetyl penicillamine (NAP) were added in the HA-stimulated cell cultures. Carboxy PTIO, a NO scavenger, or L-NIO, an eNOS inhibitor, was added in the HA-stimulated cell cultures in the presence of SNAP. Some of the cells were pre-coated with anti-human integrin αV antibody prior to incubate on HA with SNAP. Cell proliferation was assessed by colorimetric assay. The results showed that concentration up to 20 μM of SNAP, but not NAP, modulates HA-stimulated osteoblast proliferation in a dose dependent mechanism. The modulatory effect of SNAP on HA-stimulated cell proliferation was abolished by carboxy PTIO but not affected by L-NIO. The effect of SNAP on HA-stimulated cell proliferation was also eliminated by anti-integrin αV antibody. The results of the present study suggest, therefore, that low concentration of exogenous NO may up-regulate HA-stimulated osteoblast proliferation independent on osteoblast-derived NO but dependent on the interaction of osteoblast-derived integrin αV and HA surface.

Keywords: eNOS; Hydroxyapatite; Integrin αV; exogenous Nitric oxide; Osteoblasts; SNAP

Introduction

Hydroxyapatite (HA) is known as a ceramic material widely used for orthopaedic and dental implants, since this biomaterial has ability to stimulate osteoblast functions *in vitro* and *in vivo* [1]. However, the exact mechanism by which HA induces osteoblast functions remain unclear. Previous studies indicated that osteoblasts attach on HA surfaces via integrin molecules, leading to produce growth factors which in turn determine cell spreading and proliferation on the surface of protein-coated HA [2,3].

Nitric oxide (NO) is a gaseous molecule synthesized from L-arginine under catalization of nitric oxide synthase (NOS) and it plays a crucial role on many human biological systems [4]. Three isoforms of NOS, i.e., neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3), are recognized [5]. Human osteoblasts have been shown to express all NOS isoforms [6]. However, others found that osteoblasts expressed eNOS and iNOS molecules only [7,8]. It appears to suggest that iNOS and eNOS may play a role in bone inflammation and physiology, respectively [9].

The exact mechanism(s) by which NO regulates the bone formation is not well understood. Indeed, the effect of NO on osteoblast functions may be dependent on the concentration of this gaseous molecule. Adding low concentration of exogenous NO on the osteoblast cultures may augment cell proliferation [10], whereas concentration of exogenous NO higher than 1mM may lead to osteoblast apoptosis, perhaps, via the activation of Bcl2 protein [11]. Our previous study revealed that HA-induced osteoblast proliferation was regulated by endogenous NO in an integrin αV molecule and eNOS dependent mechanism (W. Sosroseno, *et al.* Submitted for publication). Therefore, the aim of the present study was to determine the effect of low concentration of exogenous NO on HA-induced human osteoblast proliferation.

Materials and methods

The HA discs (9% porosity and sintered at 1200°C), a kindly gift from Dr. Radzali Othman (School of Materials and Mineral Resources Engineering, USM, Malaysia), were cut into pieces with 2 X 2 X 2 mm^3 in size and subsequently autoclaved. In all experiments, each of the HA disc was placed in the well of 96-well plates (Corning, NY, USA).

An human osteoblast-like cell line, HOS cells, were 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 (1 X 10^6 cells/ml) was prepared in the above medium. Two hundred microliters of cell suspension containing 2 X 10^5 cells/well were plated on the surface of the HA-disc and incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2. Medium only and cell suspension plated in the wells were used as controls. S-nitroso acetyl penicillamine (SNAP), an NO donor, and nitroso acetyl penicillamine (NAP) were added in the HA-stimulated cell cultures. In the other experiments, The HA-stimulated cells were added with 20 uM of SNAP and various concentration of carboxy PTIO, an NO scavenger. Various concentration of L-NIO, an eNOS inhibitors, were also added in the HA-stimulated cell cultures in the presence of SNAP. Carboxy PTIO, L-NIO, SNAP and NAP were purchased from Sigma. Furthermore, prior to HA exposure, the cells were incubated with various concentration of anti-human αV-integrin antibody (Santa Cruz Biotech, California, USA) for 1 hour at the room temperature. The cells were then washed 3 times and then cultured on the HA disc as above. All cultures were in triplicates and incubated for 3 days after which cell proliferation was determined as described below.

Cell proliferation was determined by a colorimetric assay using crystal violet as a dye, as previously described [12]. The optical density was read at wavelength of 540 nm using μQuant spectrophotometer (Biotek-Instrument, Inc., Vermont, USA). The results were subtracted by the optical density reading of medium only.

The results were analyzed using a one-way analysis of variance followed by Fischer's least square differences with a statistical software package (SPSS co., Chicago, USA).

Results

Effect of SNAP and NAP on HA-stimulated osteoblast proliferation is displayed in Fig. 1. No significant difference between HA-stimulated cell proliferation with 5 μM of SNAP or without SNAP could be oserved ($p>0.05$). However, the addition of 10 μM of SNAP increased HA-stimulated cell proliferation as compared with that without SNAP ($p<0.05$). In fact, HA-stimulated cell proliferation added with 20 μM of SNAP was higher than that with 10 μM of SNAP ($p<0.05$). Increased concentration of NAP did not alter HA-stimulated cell proliferation ($p>0.05$).

As seen in Fig. 2, increased concentration of carboxy PTIO steadily decreased HA-stimulated cell proliferation added with SNAP as compared with the same cell cultures without additional of SNAP ($p<0.05$). HA-stimulated cell proliferation in the presence of 20 μM of SNAP and 10 μM of carboxy PTIO was lower than that with SNAP alone ($p<0.05$). Indeed, adding 100 μM of carboxy PTIO in the HA-stimulated cells in presence of SNAP induced cell proliferation lower than the cultures of cells alone ($p<0.05$).

When the HA-stimulated osteoblast cultures were added with SNAP and L-NIO, decreased cell proliferation was achieved after additional of 10 μM of L-NIO as compared with the same cultures without or with 1 μM of L-NIO ($p<0.05$) (Fig. 3). The proliferation of HA-stimulated osteoblasts in the presence of SNAP and 100 μM

of L-NIO was still higher than that of the cultures of HA-stimulated cells without SNAP or cells alone ($p<0.05$).

When osteoblasts were pre-coated with 10 but not 5 μg of anti integrin αV antibody prior to incubation of HA surface, cell proliferation in the presence of SNAP was lower than the cells with SNAP but without pre-coating with antibody ($p<0.05$) (Fig. 4). Coating the cells with 20 μg of antibody led to induce lower HA-stimulated cell proliferation with SNAP than that without SNAP but higher than proliferation of cells alone ($p<0.05$).

Discussion

The results of the present study showed that proliferation of human osteoblast-like cells stimulated with HA was significantly increased in presence of exogenous NO at low concentration in a dose dependent mechanism. These results are not surprising since previous reports showed that exogenous NO did stimulate increased osteoblast proliferation [10]. Previous studies indicated that NO directly acts on osteoblast proliferation by enhancing cGMP levels [10,13,14]. Other found that increased osteoblast functions induced by exogenous NO may partly mediated by the release of prostaglandin E_2 (PGE_2) [15]. Whether or not augmented HA-stimulated osteoblast proliferation by exogenous NO in the present study may be mediated by increased levels of cGMP or release of this cytokine remains to be further clarified, since the present study did not determine the levels of cGMP and PGE_2. In contrast, a previous report demonstrated that concentration of SIN-1, a NO donor, as low as 10 μM suppressed osteoblast proliferation [16]. The exact reason to explain this discrepancy is unclear. Despite being known as one of NO-releasing agents, SIN-1 also stimulates peroxynitrite production which in turn induces cell death [17,18]. In fact, concentration of SIN-1 up to 1 mM did only induce peroxynitrite but not NO production [19]. Therefore, a possibility that suppressed osteoblast

functions by SIN-1 as described by a previous study [16] may be mediated by peroxynitrite but not NO can not be ruled out.

The results of the present study also showed that increased HA-stimulated osteoblast proliferation by exogenous NO was abolished by carboxy PTIO. Carboxy PTIO oxidizes NO to generate nitrogen dioxide, NO_2, which may either react further with NO to form N_2O_3 reacting rapidly with water to yield NO_2^-, or may dimerize to form N_2O_4, spontaneously dismutating to yield NO_2^- and NO_3^- [20,21]. The present study suggests, therefore, that augmented HA-stimulated osteoblast proliferation may be regulated by NO.

It should be borne in mind, however, that osteoblasts are able to produce NO which in turn regulates osteoblast functions (9). We also showed that HA-stimulated osteoblast proliferation and NO production were an eNOS dependent mechanism (W. Sosroseno, *et al.*, Submitted for publication), suggesting that endogenous NO may regulate HA-stimulated osteoblast proliferation. In order to eliminate a possibility that the endogenously produced NO may interfere the effects of exogenous NO in HA-stimulated osteoblast proliferation, the HA-stimulated cell cultures were added with NO donor and L-NIO, an eNOS inhibitor. The results indicated that HA-stimulated cell proliferation after adding SNAP and 100 μM of L-NIO was still higher than that without the presence of SNAP, suggesting that exogenous NO alone may indeed up-regulate HA-stimulated osteoblast proliferation without interference of osteoblast-derived NO. The results of the present study support the previous findings that exogenous NO may directly facilitate the osteoblast proliferation and differentiation (13,14).

Our previous study indicated that HA-stimulated osteoblast proliferation and NO production was mediated by integrin αV molecules (W. Sosroseno, *et al.*). Whether increased HA-stimulated osteoblast proliferation by exogenous NO was dependent on the presence of this integrin molecule was then tested. The results

showed that the effect of exogenous NO on HA-stimulated osteoblast proliferation was abolished in the presence of anti-integrin αV antibody, suggesting that regardless of the presence of exogenous NO, integrin αV molecules remain the key molecule of osteblasts to attach and proliferate on HA surface as previously described (22). If so, it would seem plausible that exogenous NO may amplify the proliferative signals generated from osteoblast-derived integrin αV molecules which may interact with ECM absorbed on the surface of HA. This notion is also supported by the results of the present study that the proliferation of unbound osteoblasts due to pre-coating with anti-integrin αV antibody was still induced by exogenous NO. This assumption remains to be investigated further, however.

In summary, the present study showed that exogenous NO up-regulates HA-stimulated osteoblast proliferation. The effect of exogenous NO on HA-stimulated osteoblast proliferation was abolished by NO scavenger but not by L-NIO. The effect of exogenous NO on HA-stimulated osteoblast proliferation was dependent on the presence of integrin αV molecules. Therefore, the results of the present study suggest that exogenous NO may indeed modulate HA-stimulated osteoblast proliferation in an independent on endogenously synthesized NO but dependent on the interaction of osteoblast-derived integrin αV molecule and HA.

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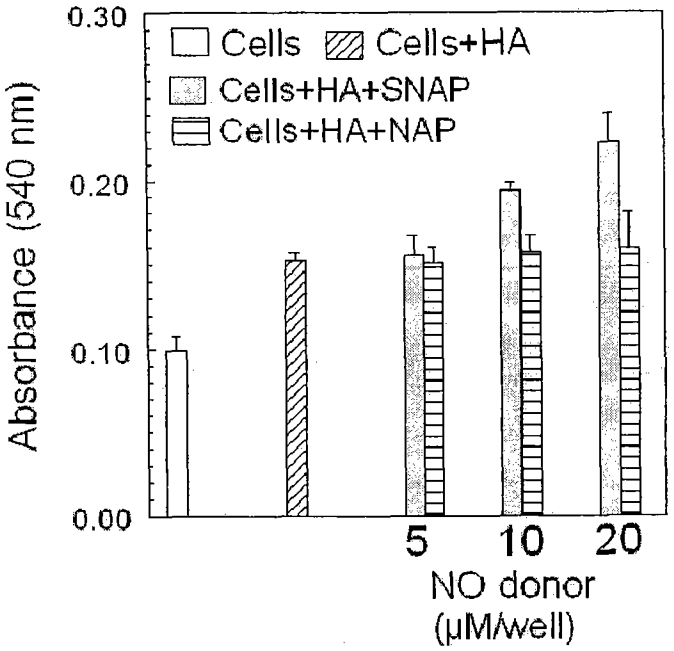
Legend of figures

Fig. 1. Effect of exogenous NO (SNAP and NAP) on mean and standard deviation of HA-induced human osteoblast proliferation. The HA-stimulated osteoblast cultures were added with SNAP or NAP and incubated for 3 days.

Fig. 2. Effect of carboxy PTIO-oxidized exogenous NO on mean and standard deviation of HA-induced human osteoblast proliferation. The HA-stimulated osteoblast cultures were added with 20 μM of SNAP and various concentration of carboxy PTIO and the cultures were incubated for 3 days.

Fig. 3. Mean and standard deviation of HA-induced human osteoblast proliferation following addition of exogenous NO and L-NIO, an eNOS inhibitor. The HA-stimulated osteoblast cultures were added with 20 μM of SNAP and various concentration of L-NIO and the cultures were incubated for 3 days.

Fig. 4. Mean and standard deviation of the proliferation of anti-integrin αV antibody-treated human osteoblasts cultured on HA in the presence of exogenous NO. Human osteoblasts were treated with anti-integrin αV antibody prior to culture on the HA surface in the presence of 20 μM of SNAP. The cultures were incubated for 3 days.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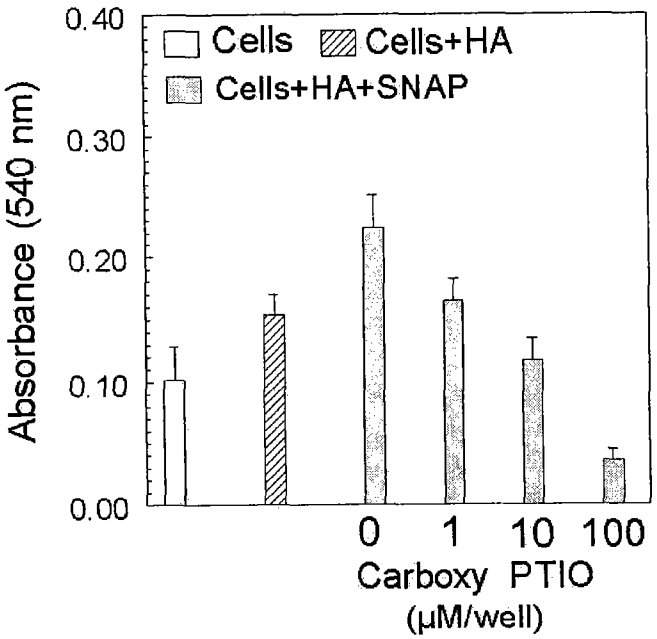
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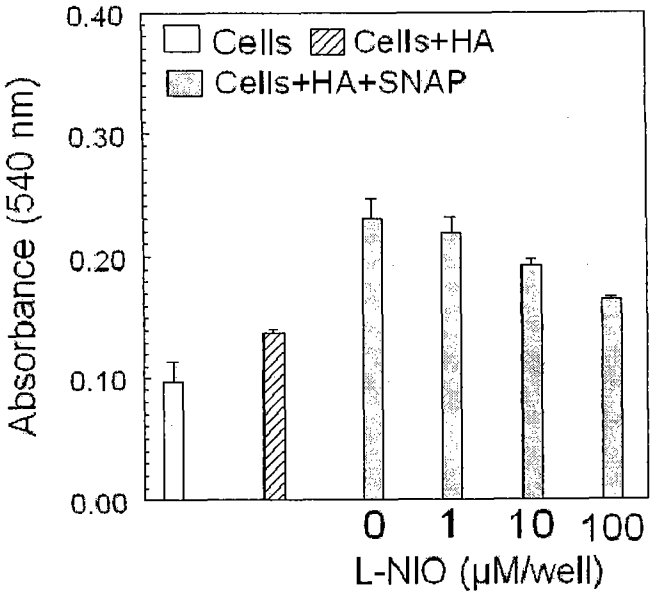
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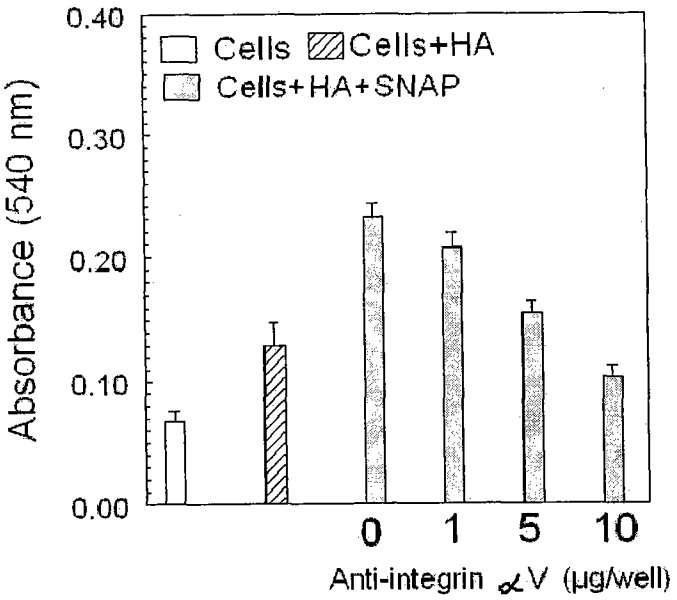
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