ORIGINAL RESEARCH

Synergism of Prostaglandin E_2 and Nitric Oxide on Human Osteoblast Proliferation Induced by Hydroxyapatite

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Abstract

Aim: To assess whether osteoblast proliferation induced by hydroxyapatite (Ha) may be regulated by prostaglandin E_2 (PGE_2) and nitric oxide (NO) in a synergic fashion.

Materials and methods: Human osteoblasts (HOS cell line) were cultured onto Ha with or without nimesulide, a cyclooxygenase-2 (COX-2), and/or L-NIO, an endothelial nitric oxide synthase (eNOS) inhibitor. The cells pretreated with nimesulide and/or L-NIO were cultured onto Ha added with PGE_2 and/or *S*-nitroso acetyl penicillamine (SNAP), a NO donor. The Ha-plated cell cultures were also added with anti-PGE_2 and/or carboxy PTIO, a NO scavenger. The cell proliferation was assessed colorimetrically from the 3-day cultures. The levels of PGE_2 and NO were determined from the culture supernatants.

Results: Hydroxyapatite-induced cell proliferation was partially inhibited by nimesulide or L-NIO but fully by both inhibitors. The production of PGE_2 from the same cell cultures was inhibited fully by nimesulide but partially by L-NIO. In contrast, NO production was inhibited only by L-NIO. Partial suppression of Ha-stimulated cell proliferation by nimesulide or L-NIO was abolished by PGE_2 or NO, respectively. The combination of PGE_2 and NO donor could abrogate fully nimesulide—but partially L-NIO-mediated suppression of Ha-induced cell proliferation. Anti-PGE_2 or carboxy PTIO partially inhibited but combination of both scavengers fully suppressed the Ha-induced cell proliferation.

Conclusion: Osteoblast proliferation induced by Ha may be regulated by a synergic function of PGE_2 and NO in an autocrine fashion.

Clinical significance: The successful or failing Ha-based dental implantation may be determined by the synergic regulatory functions of the host PGE_2 and NO at the implanted sites.

Keywords: Hydroxyapatite, Nitric oxide, Osteoblasts, PGE_2, Proliferation.

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Introduction

The crucial roles of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in the regulation of bone remodeling have been well studied. Prostaglandin E_2, is a prostanoid synthesized by the action of cyclooxygenases (COX-1 and COX-2) on arachidonic acid,[1] and is a potent regulator of osteoclast and osteoblast differentiation and proliferation.[2] Interestingly, the most recent report showed that the regulatory role of PGE_2 on bone formation is via its role on sensory nerve which in turn inhibits sympathetic activity and, hence, promotes osteogenesis.[3] Nitric oxide is a gaseous molecule generated from nitric oxide synthases (NOS)—catalyzed L-arginine metabolism and is a crucial molecule on bone remodeling.[4] For example, NO was reported to directly stimulate osteoblast proliferation.[5] Interestingly, NO was able to modulate the COX-2-mediated PGE_2 production by cytokine-stimulated rat osteoblasts,[6] fluid shear stress-stimulated rat preosteoblasts,[7] and ascorbic acid-induced mouse osteoblast differentiation,[8] suggesting the existence of the NO–PGE_2 crosstalk during bone formation in physiological or pathological condition.

Hydroxyapatite (Ha) is commonly used for orthopedic and dental implants because of its potent osteoconductive properties.[9] Binding between osteoblast-derived integrin molecules and its ligand expressed by Ha covering protein layer results in the cells releasing numerous mediators, such as osteopontin, PGE_2, and transforming growth factor-β (TGF-β) which involve in bone remodeling.[10–14] Previous reports indicated that upon binding between integrin αV-expressing human osteoblasts and Ha, cell proliferation, and the production of endothelial NOS (eNOS)-catalyzed NO and COX-2-mediated PGE_2 are observed.[15,16] Furthermore, NO-regulated Ha-plated osteoblast proliferation by amplifying cell proliferation, PGE_2 production, and cAMP-PKA signal transduction pathway.[16–18] Interestingly, inhibition of eNOS activity was associated with suppression of PGE_2 production by Ha-plated osteoblasts,[18] suggesting a possible regulatory link between NO and PGE_2 in the human osteoblast proliferation stimulated with Ha. Therefore, the aim of the study was to test whether PGE_2 and NO may synergically regulate the proliferation of human osteoblasts induced by Ha.

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Materials and Methods

All materials were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated. Sintered and sterilized Ha (9% porosity) were provided by the School of Materials and Mineral Resource Engineering, USM, Malaysia.[15] Human osteoblasts (HOS cell line) was obtained from American Type Culture Collection (Rockville, MD, USA).

The culture medium containing Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin–streptomycin were used to culture the cells. One million cells in 1 mL of the culture medium containing 100 µM of nimesulide, a COX-2 inhibitor, and/or 100 µM of L-NIO, an eNOS inhibitor, were incubated for 30 minutes at room temperature as previously described.[15,18] Two hundred thousand cells in 200 µL of culture medium were cultured onto Ha with or without 10 µM of PGE_2 and/or 20 µM of SNAP, a NO donor, in the 96-well plates (Corning, NY, USA). Furthermore, 2×10^5 cells in 200 µL of cell suspension were cultured onto Ha and then, 100 pg of polyclonal anti-PGE_2 and/or 100 µM of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy PTIO), a NO scavenger, were added.[15] The cells cultured with or without Ha were served as the positive or negative control, respectively. All cultures prepared in triplicate were then incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2.

The cell proliferation was determined as previously documented.[15] Briefly, the cells were treated with 100 µL of 20% methanol for 10 minutes followed by 100 µL of 0.5% crystal violet for 5 minutes. After washing, the cells were treated with 100 µL of 0.5% crystal violet for 5 minutes for releasing the dye. The solution was read spectrophotometrically at 540 nm (Biotek-Instrument, Inc., Winooski, Vt). After subtracting from the reading of medium only, the results were expressed as absorbance unit.

The end product of NO, i.e., nitrite, was determined by the Griess reagent containing 1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid.[15] Equal volume (100 µL) of culture supernatant and the reagent were mixed and then read spectrophotometrically at 540 nm (Biotek-Instrument, Inc., Winooski, Vt). Sodium nitrite was used to prepare a standard curve.

The levels of culture supernatant PGE_2 was assessed by using a commercial kit (Cayman Chemical Co., Ann Arbor, MI, USA). Statistically, one-way analysis of variance followed by Fischer's least square differences (SPPS, Chicago, Ill, USA) was used to analyze the data.

Results

The Effect of Nimesulide and/or L-NIO

Hydroxyapatite did induce proliferation of osteoblasts as compared with the negative control ($p < 0.05$), as shown in Table 1. Similarly, PGE_2 and NO were simultaneously produced by cells induced by Ha ($p < 0.05$). Nimesulide or L-NIO did cause Ha-stimulated cell proliferation lower than the positive but higher than the negative control ($p < 0.05$). Interestingly, while nimesulide inhibited PGE_2 but not NO production ($p < 0.05$), L-NIO suppressed both NO and PGE_2 by Ha-stimulated cells ($p < 0.05$). The Ha-induced cell proliferation and production of both PGE_2 and NO were abolished by combination of nimesulide and L-NIO ($p < 0.05$).

Furthermore, nimesulide significantly decreased cell proliferation induced by Ha but exogenous PGE_2 or NO partially abolished this nimesulide-mediated suppression of cell proliferation ($p < 0.05$) (Fig. 1). Interestingly, the inhibitory effect of nimesulide on Ha-induced cell proliferation was abrogated by combination of exogenous PGE_2 and NO as compared with the positive control ($p < 0.05$). Moreover, an eNOS inhibitor (L-NIO) in the Ha-plated cell cultures led to the cell proliferation lower than the positive control ($p < 0.05$) (Fig. 2). The suppressive L-NIO on cell proliferation could be partially overcome by exogenous PGE_2 ($p < 0.05$) but completely abrogated by exogenous NO ($p < 0.05$). Interestingly, when the cells were pretreated with L-NIO, added with combination of PGE_2 and NO donor, and then cultured onto Ha, the cell proliferation was lower than the positive control but still higher than the negative control ($p < 0.05$).

The effect of simultaneous COX-2 and eNOS inhibition by their respective inhibitor on Ha-induced cell proliferation was examined and the results are depicted in Figure 3. The proliferation of cells induced by Ha was suppressed by combination of nimesulide and L-NIO as compared with both positive and negative control ($p < 0.05$). The suppressive effect of these inhibitors on the cell proliferation induced by Ha was partially abrogated by either exogenous PGE_2 or NO but completely abolished by combination of exogenous PGE_2 and NO ($p < 0.05$).

Effect of Anti-PGE_2 and/or Carboxy PTIO

Anti-PGE_2 antibody or carboxy PTIO induced lower osteoblast proliferation stimulated by Ha than the positive but higher than the negative control ($p < 0.05$) (Fig. 4). The proliferation of cells treated with the combination of anti-PGE2 antibody and carboxy PTIO and then cultured onto Ha was lower than that of the positive control ($p < 0.05$) and was, in fact, comparable with that of the negative control ($p > 0.05$).

Discussion

The present study showing the cell proliferation and both PGE_2 and NO production by osteoblasts induced by Ha are not unexpected and have been reported.[13,15,18] These results are also supported by the fact that ultrasound-induced osteoblasts produce PGE_2 and NO.[19] Furthermore, when the production of PGE_2 and NO was inhibited by cyclooxygenase-2 and eNOS inhibitor respectively, the cell proliferation induced by Ha was partially decreased, suggesting that PGE_2 or NO alone may individually act as a partial regulator

Table 1: Effect of COX-2 and/or eNOS inhibitor on the cell proliferation and both prostaglandin E_2 and nitric oxide production by hydroxyapatite-stimulated osteoblasts

Cultures	*Cell proliferation (absorbance unit) (mean + SD)*	*PGE_2 (pg/mL) (mean + SD)*	*Nitrite (µM) (mean + SD)*
Cells	0.094 + 0.002	80.667 + 2.867	5.233 + 0.499
Cells + Ha	0.261 + 0.005#	141.667 + 3.858#	14.483 + 1.375#
Cells + Ha + L-NIO	0.134 + 0.009#*	98.333 + 6.599#*	0.950 + 0.043#*
Cells + Ha + nimesulide	0.140 + 0.007#*	1.133 + 0.275#*	14.543 + 0.903#
Cells + Ha + nimesulide + L-NIO	0.082 + 0.007*	1.197 + 0.200#*	0.933 + 0.074#*

Note: Ha, hydroxyapatite; nimesulide, cyclooxygenase (COX)-2 inhibitor (100 µM); L-NIO, an endothelial nitric oxide synthase (eNOS) inhibitor (100 µM)
#Significant difference to the negative control (cells only) at $p < 0.05$
*Significant difference to the positive control (cells + Ha) at $p < 0.05$

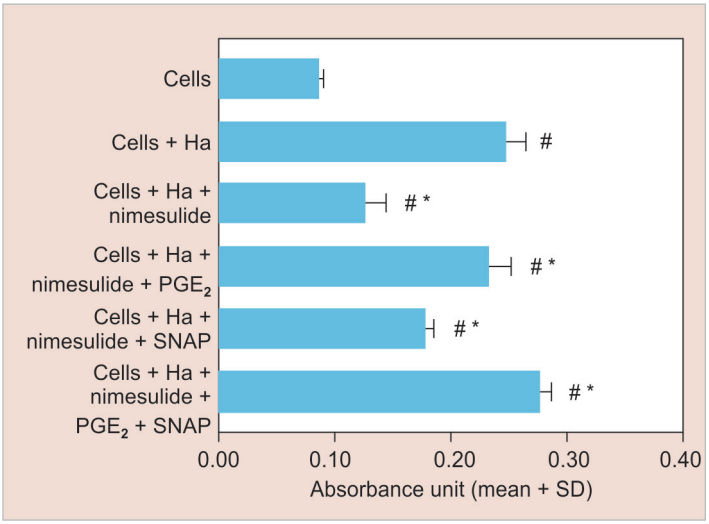

Fig. 1: Effect of exogenous prostaglandin E_2 and/or nitric oxide on the proliferation of human osteoblasts treated with a cyclooxygenase (COX)-2 inhibitor (nimesulide) and stimulated with hydroxyapatite

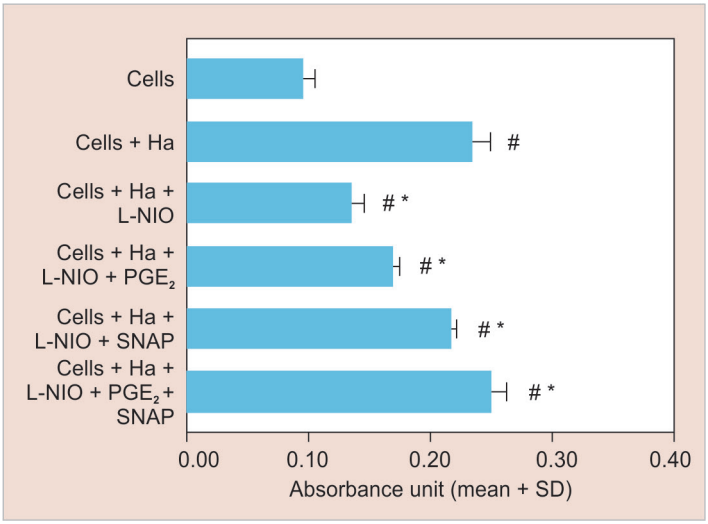

Fig. 2: Effect of exogenous prostaglandin E_2 and/or nitric oxide on the proliferation of human osteoblasts treated with an endothelial nitric oxide synthase inhibitor (L-NIO) and stimulated with hydroxyapatite

on the human osteoblast proliferation stimulated with Ha. This notion is particularly evidenced by the fact that when complete inhibition of Ha-induced osteoblast proliferation could only be achieved by concurrent inhibition of COX-2 and eNOS activity in the present study, indicating requirement of both PGE_2 and NO on the proliferation of human osteoblasts stimulated with hydoxyapatite. The explanation on these results is not well understood. Several studies indicated a possible regulatory link of PGE_2 and NO released by stimulated osteoblasts since activated osteoblasts produced NO which in turn modulates the secretion of COX-2-mediated PGE_2 and alters osteoblast functions.[6–8] Hence, a possible link between NO and PGE_2 in the regulation of human osteoblast proliferation induced by Ha in the present study should not be ruled out.

That the suppressive effect of COX-2 proliferation of human osteoblasts induced by Ha could be abrogated fully by PGE_2 but partially by NO donor may validate that PGE_2 alone may only partially regulate the Ha-induced cell proliferation. Similarly, NO seems also to partially involve in the regulation of Ha-induced cell proliferation, since NO donor but not PGE_2 recover the suppressive effect of eNOS inhibitor on the cell proliferation. These results could be supported by the fact that PGE_2 and NO are individually potent promoter on implant material-induced osteoblast proliferation.[17,20] It should be noted, furthermore, that after adding both exogenous PGE_2 and NO into the Ha-plated cell cultures, the proliferation of cells preincubated with COX-2 inhibitor was accelerated but that preincubated with eNOS inhibitor was inhibited. These results may imply that combination of low PGE_2 and high NO levels may accelerate the Ha-induced cell proliferation. Conversely, combination of high PGE_2 and low NO may inhibit the Ha-induced cell proliferation. The reciprocal levels of PGE_2 and NO in the surrounding tissues of implanted site could also be found in the experimental aseptic implant loosening in sheep.[21] In this study, the levels of NO in the stable implants were higher than those of PGE_2 but reversed profile occurred in the failed implants. The exact mechanism(s) to explain these phenomena remains unclear, however. NO was found to upregulate the cell proliferation and

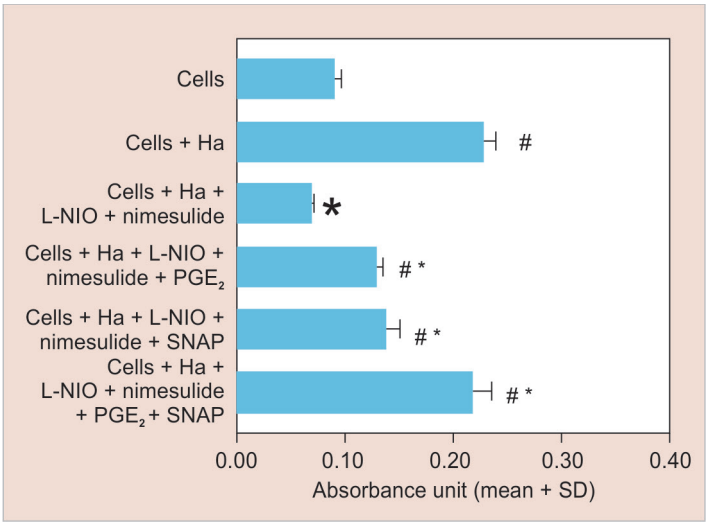

Fig. 3: Effect of exogenous prostaglandin E_2 and/or nitric oxide on the proliferation of human osteoblasts treated with the combination of nimesulide and L-NIO and stimulated with hydroxyapatite

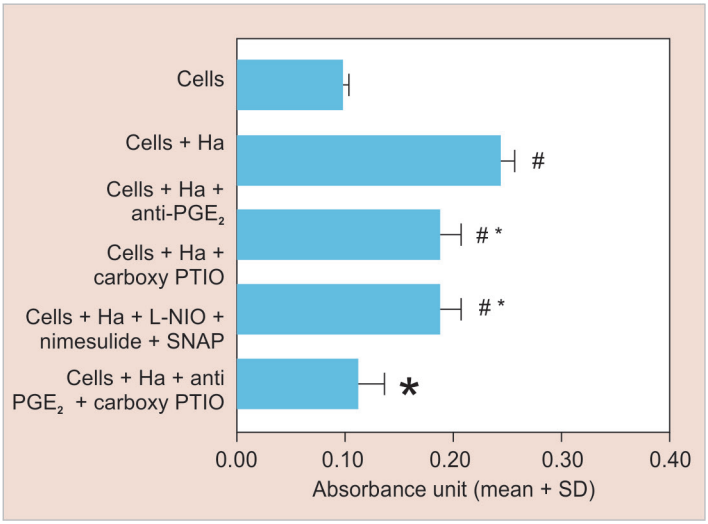

Fig. 4: Effect of anti-prostaglandin E_2 and/or a nitric oxide scavenger (carboxy PTIO) on the proliferation of human osteoblasts stimulated with hydroxyapatite

PGE_2 production by Ha-stimulated human osteoblasts.[15–17] Previous reports demonstrating that nitric oxide enhances the production of PGE_2 by inflammatory cytokine-stimulated osteoblasts[22] and that increased PGE_2 levels seem to be responsible for the inhibition of Ha-induced osteoblast functions[13] may be in accordance with the results of the present study.

When both COX-2 and eNOS were simultaneously inhibited, the proliferation of osteoblasts induced by Ha could only be fully recovered by combination of exogenous NO and PGE_2 but neither one of them, validating the view that the proliferation of human osteoblasts induced by Ha may require simultaneous action of both endogenous PGE_2 and NO. Indeed, that combination of anti-PGE_2 antibody and carboxy PTIO inhibited osteoblast proliferation induced by Ha clearly indicates that PGE_2 and NO do play a synergic role in Ha-induced human osteoblast proliferation in an autocrine fashion. The NO–PGE_2 crosstalk via peroxynitrite-mediated pathway or *S*-nitroxylation of COX enzymes in different types of cells has been well documented[23] and may support the present study. Alternatively, NO released by Ha-induced osteoblasts may amplify intracellular activation of the cAMP-PKA pathway which may in turn increase PGE_2 production[18] and hence, upregulate the cell proliferation. The fact that increased PGE_2 production was associated with increased the cAMP-PKA pathway which might then upregulate bone remodeling[24] may also be in the line of this notion. However, this assumption requires to be further studied.

The clinical implication of the present study in orthopedic and dental implantation with Ha in humans needs to be further studied. Examination on the site of dental implants showed that low levels of PGE_2 and NO in healthy implant are associated with bone formation remodeling but high levels of these mediators are observed in the sites of implant failure.[25–27] Therefore, one may assume that both PGE_2 and NO released by osteoblasts and other cells at the surrounding implanted materials may regulate the bone remodeling in a synergic fashion and that their combined concentration may determine whether the orthopedic or dental implantation is successful or failed treatment.

Conclusion

The proliferation of human osteoblast induced by Ha could be inhibited partially by nimesulide or L-NIO but fully by combination of these inhibitors. In fact, only nimesulide, but not L-NIO-mediated suppression of Ha-stimulated cell proliferation could only be abrogated by combination of PGE_2 and NO. The suppression of Ha-induced cell proliferation due to simultaneous inhibition of COX-2 and eNOS activity could only be abolished by combined PGE_2 and NO but neither one of them. The cell proliferation stimulated with Ha was inhibited partially by anti-PGE_2 antibody or carboxy PTIO alone but fully by combination of both scavengers. Therefore, the present study suggests that human osteoblast proliferation induced by Ha may be regulated by a synergic function of PGE_2 and NO in an autocrine fashion.

Clinical Significance

This *in vitro* study may imply that the successful or failing Ha-based dental implantation may be determined by the synergic regulatory functions of the host PGE_2 and NO at the implanted sites.

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