ORIGINAL ARTICLE

Effects of nitric oxide on the proliferation and differentiation of human periodontal ligament cells

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Objective: This study evaluated the effects of nitric oxide (NO) on the proliferation and differentiation of human periodontal ligament cells involved in orthodontic tooth movement. **Methods:** A range of concentrations of sodium nitroprusside (SNP), a NO donor, were administered to samples of human periodontal ligament cells, followed by measurement of cell viability, alkaline phosphatase (ALP) activity, and expression of osteonectin and bone sialoprotein. **Results:** Cell viability, ALP activity and the expression of osteonectin and bone sialoprotein were increased in human periodontal ligament cells treated with SNP concentrations of < 0.2 mM compared with controls, but were decreased with SNP concentrations of > 1.0 mM. **Conclusion:** NO has a biphasic effect on proliferation and differentiation in human periodontal ligament cells, with a stimulatory effect at low concentrations and an inhibitory effect at high concentrations. **(Korean J Orthod 2006;36(6):465-73)**

Key words: Nitric oxide, Human periodontal ligament cells, Growth and differentiation

INTRODUCTION

Tooth movement is generated in response to orthodontic force that results in bone resorption on the compression side and bone apposition on the tension side. Human periodontal ligament (PDL) cells are involved in tooth movement during this bone remodeling procedure. There have been numerous studies on the physiological and biochemical response of human PDL cells when exposed to mechanical stress.[1-6]

The mechanism involved in tooth movement is not completely understood, although it was reported recently that cultured human PDL cells produced small amounts of nitric oxide (NO) spontaneously. This production of NO was increased markedly in human PDL cells stimulated mechanically by cyclic tension forces[5] or hydraulic pressure compared to the magnitude of clinically applied orthodontic force.[6]

NO is a free radical gas produced from the amino acid L-arginine by the nitric oxide synthase (NOS) group of enzymes. Its roles are so diverse that it acts as an endothelium-derived relaxing factor, a neuromodulator, and an immunologic mediator in the cardiovascular, nervous, and immune systems. NO is also known to be an important signaling molecule in bone. Several studies in the last few years have demonstrated that bone cells express NOS enzymes and can produce NO, and it is now clear that NO plays an important role as a paracrine and autocrine mediator in bone cells in response to diverse stimulations such as mechanical strain,[7] pro-inflammatory cytokines[8] and hormones.[9]

Recent research suggests that NO exerts biphasic

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effects on bone cell activity, with high NO concentrations inhibiting bone resorption and low concentrations potentiating bone resorption, actions that may be essential for normal osteoclast function.[10] Similarly, growth and differentiation of osteoblasts are inhibited by high NO concentrations which may be responsible in part for the inhibitory effects of pro-inflammatory cytokines on bone formation. In contrast, lower concentrations of NO may play a role in the regulation of normal osteoblast growth and mediate the effects of estrogens on bone formation.[11,12]

In earlier studies, collagen synthesis analysis, von Kossa staining, alkaline phosphatase (ALP) activity, osteonectin (ON), osteocalcin (OC) and bone sialoprotein (BSP) expression have usually been used as markers of osteoblast-like differentiation.[11-13]

Although NO produced in human PDL cells by mechanical stimulation, such as orthodontic force, may be involved in bone remodeling, the role of NO in human PDL cells has not been clarified. To further understand the role of NO in human PDL cells and its clinical relevance in orthodontic tooth movement, we examined the effects of NO on the proliferation and differentiation of human PDL cells by measuring cell viability, ALP activity and ON and BSP expression as markers of differentiation.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other cell culture reagents were obtained from Lifetechnologies (Grand Island, NY, USA). Sodium nitroprusside (SNP) was purchased from Sigma Chemical Co (St. Louis, MO, USA). Rabbit polyclonal antibody to ON was obtained from Biodesign International (Saco, Maine, USA) and rabbit polyclonal antibody to BSP was purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Cell culture

Human PDL cells were obtained from explant cultures of human PDL tissue obtained from healthy premolar teeth extracted for orthodontic reasons.[14-16] Tissue attached to the mid-third of the root surface was removed with a surgical scalpel. The cultures were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. All the experiments were performed using cells within 6-8 passages.

MTT cell viability assay

The MTT colorimetric assay was developed to monitor mammalian cell survival and proliferation. The number of viable cells was determined based on the reduction of MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (Sigma, St louis, MO, USA) by mitochondrial dehydrogenase in live cells with the resulting formation of blue formazan crystals. The MTT solution was prepared as a 0.5 mg/mℓ stock solution in PBS and was prepared fresh prior to each experiment. Each experimental group was composed of 4 samples for statistical analysis. Briefly, 4×10^4 cells were seeded to a 24-well plate in 1 mℓ of medium and left overnight to attach. Serial dilutions of NO in 1 mℓ volume were added, and the cells incubated for either 1, 2, 3 or 7 days. After incubation, the medium was aspirated and 300 μℓ of MTT solution (0.5 mg/μℓ in PBS) was added to each well and incubated for a further 4h at 37℃. The MTT solution was aspirated in each well and the formazan reaction products dissolved in 200 μℓ DMSO. The optical density of the formazan solution was then measured on an ELISA plate reader at 540 nm.

Analysis of differentiation of human PDL cells

In the differentiation experiments, confluent PDL cells were incubated for 24h in DMEM/10% FBS containing 50 μg/ml ascorbic acid, 10 mM Sodium-glycerophosphate and 10^{-7}M dexamethasone. The cells were then subjected to NO at each concentration for 3, 7 or 14 days. Cultures without 50 μg/ml ascorbic acid, 10 mM sodium glycerophosphate and 10^{-7}M dexa-

methasone were used as negative controls and were also subjected to NO at the same each concentrations for 3, 7 and 14 days. ALP activity, and ON and BSP expression of the cell lysate were measured as markers of PDL cell differentiation.

ALP activity

Each experimental group for measurement of ALP activity was composed of 4 samples for statistical analysis. Conditioned medium samples were collected on days 1, 3, and 7. The periodontal cells were scraped into cold PBS and sonicated in an ice-cold bath with a sonicator cell disrupter (Heat system-Ultrasonics, Plainview, USA). ALP activity in the supernatant was determined by the method of Lowry et al.[8] using p-nitrophenyl phosphate as the substrate. The assay system for ALP consisted of 0.1 M glycine NaOH buffer (pH 10.4), 15 mM p-nitrophenyl phosphate (p-NPP; Sigma Diagnostics, USA), 0.1% triton X-100/ saline. The samples were incubated at 37℃ for 30 min and the reaction was stopped by the addition of 0.1 M NaOH. Absorbance was measured at 410 nm using an ELISA plate reader (Beckman DU-650, Fullerton, CA, USA).

A 50 $\mu\ell$ aliquot of protein of the extracted samples was pipetted into each tube, followed by the addition of bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA) prepared at a ratio of 50:1. The samples were then incubated at 37℃ for 30 min and the optical density measured at 562 nm.

Western Blot Analysis

ON and BSP were identified in the periodontal ligament by immunoblotting with the respective polyclonal antibodies. Briefly, the periodontal cells were solubilized with SDS-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, and 0.5% Triton X-100, 2 mM phenylmethyl-sulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice. The cell lysates were then centrifuged at 12,000 g at 4℃ and the protein concentrations determined with Bradford reagent (Bio-rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as the standard. Equivalent amounts of total protein per sample of cell extracts were run on 12% SDS-PAGE. SDS-PAGE was carried out by method of Weber and Osborn with the gels incorporating a 7.5% stacking gel in 50 mM/L phosphate buffer containing 0.1% SDS at pH 7.0. Proteins in the gel were stained with silver or transferred electrophoretically to a PVDF membrane (350 mA, 5 hours). The transfer medium was 25 mM/L Tris, 192 mM/L glycine, pH 8.3, containing 20% methanol. The membranes were blocked with 3% non-fat dry milk for 1.5 h and then incubated at 37℃ for 1.5 h with either anti-ON (1:2000 dilution) or and anti-BSP (1:2000 dilution) polyclonal antibodies. After washing with PBS, the membranes were incubated with each anti-rabbit (ON, BSP) IgG in PBS for 1 h at 37℃. The membranes were then washed again and the reactive bands were visualized using enhanced chemiluminescence.

Statistical analysis

Statistical evaluation of the results of the MTT assay and ALP activity was performed with analysis of variance, followed by Tukey's multiple comparison test. Significance was inferred when the p value was <0.05.

RESULTS

Effects of SNP on viability of human PDL cells

As the relative sensitivity of human PDL cells towards NO is not known, we first investigated whether NO affected cell viability in human PDL cells. Figure 1 shows the dose-dependent effects of a range of SNP concentrations (0.05 ~ 1.0 mM) on cell viability measured by the MTT assay following treatment for 1, 2, 3 and 7 days.

When the human PDL cells were exposed to SNP for up to 2 days, there were no significant differences between the experimental groups and controls. However, SNP treatment ranging from 0.05 to 0.5 mM stimulated the growth of human PDL cells after 3 days of culture. After 7 days incubation, cell viability was decreased dose-dependently by treatment with SNP at

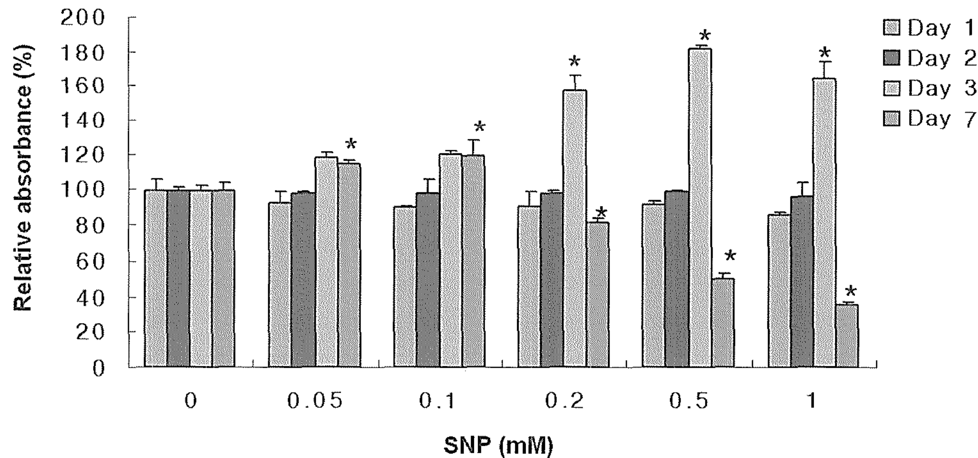

Fig 1. Effects of SNP on cell viability of the human PDL cells by MTT assay. *SNP,* sodium nitroprusside; $^{*}p < 0.05$ (compared with control).

Table 1. Effects of SNP on ALP activity of human PDL cells. ALP activities of human PDL cells treated with various SNP in the positive group (with dexamethasone)

	Concentration of SNP (mM)					
	C^+	0.05	0.1	0.2	0.5	1
Day 1	0.10 ± 0.06	0.13 ± 0.04	0.14 ± 0.04	0.15 ± 0.07	0.13 ± 0.03	0.05 ± 0.03
Day 3	0.27 ± 0.01	0.63 ± 0.08*	0.63 ± 0.12*	0.64 ± 0.16*	0.38 ± 0.15	0.27 ± 0.00
Day 7	0.36 ± 0.05	0.37 ± 0.02	0.32 ± 0.02	0.31 ± 0.05	0.10 ± 0.00*	0.09 ± 0.01*

Results are expressed as mean ± SD, C^+: positive control. *Statistical significance among subgroup difference was tested by Tukey's multiple comparison test at a significance level of $p < 0.05$.

concentrations of > 0.2 mM.

The stimulating effect of NO on human PDL cell replication was achieved only in the presence of low concentrations of SNP. Conversely, high concentrations of SNP caused a dose-dependent decrease in cell viability after 7 days incubation.

Effects of SNP on mineralization of human PDL cells

To examine the effects of SNP-induced NO production on differentiation of human PDL cells, we investigated ALP activity and ON and BSP expression as differentiation markers.

Effects of SNP on ALP activity

To examine the effects of SNP on ALP activity in human PDL cells, the cells were treated with 0.05 ~ 1.0 mM SNP for 1, 3 and 7 days. The SNP at each concentration was diluted in DMEM/10% FBS with 50 μg/ml ascorbic acid, 10 mM Sodium-glycerophosphate and 10^{-7}M dexamethasone in the positive control group, and without supplement in the negative control group.

The effects of SNP on ALP activity in human PDL cells are summarized in Tables 1 and 2. After exposure to SNP, ALP activity was increased in human PDL cells treated with dexamethasone compared to the non-treated group.

In the dexamethasone-treated group, ALP activity increased to a greater extent with SNP concentrations

Table 2. Effects of SNP on ALP activity of the human PDL cells. ALP activities of human PDL cells treated with various SNP in the negative group (without dexamethasone)

	Concentration of SNP (mM)					
	C⁻	0.05	0.1	0.2	0.5	1
Day 1	0.01 ± 0.00	0.14 ± 0.03	0.12 ± 0.03	0.11 ± 0.03	0.12 ± 0.02	0.11 ± 0.03
Day 3	0.12 ± 0.02	0.26 ± 0.01*	0.07 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.00
Day 7	0.23 ± 0.06	0.25 ± 0.05*	0.22 ± 0.00	0.19 ± 0.01	0.21 ± 0.08	0.17 ± 0.01

Results are expressed as mean ± SD, C⁻: negative control. * Statistical significance among subgroup difference was tested by Tukey's multiple comparison test at a significance level of $p < 0.05$.

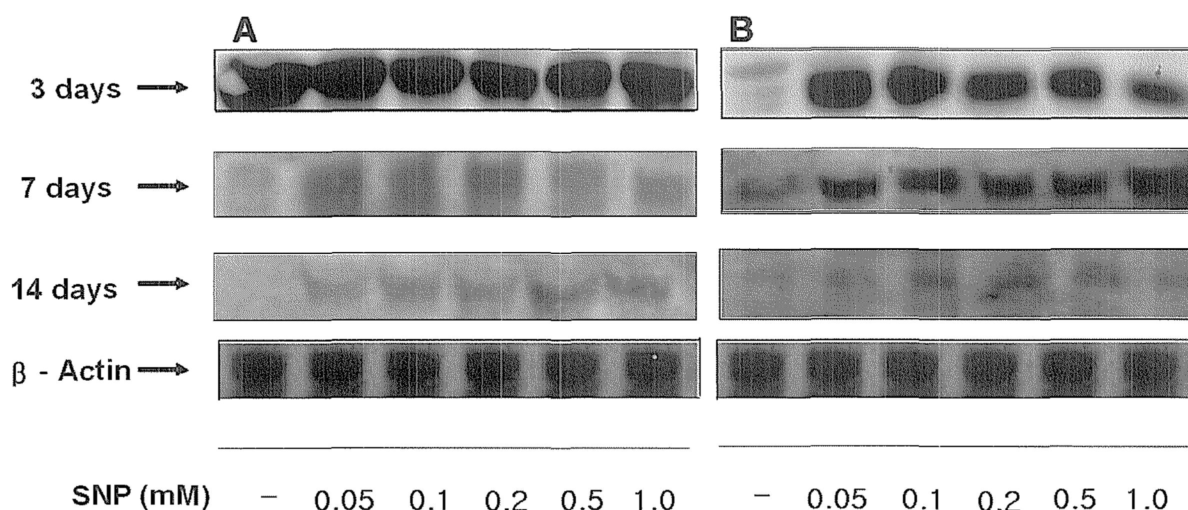

Fig 2. Western blot analysis of ON expression in human PDL cells cultured with or without dexamethasone and different concentrations of NO for 3, 7, and 14 days. **A,** Expression of ON in the positive group (with dexamethasone); **B,** Expression of ON in the positive group (without dexamethasone). The protein fraction was extracted, electrophoresed and transferred to membrane and blotted with antibody. β-Actin was used as the control. These data are representative of three independent experiments. *SNP,* sodium nitroprusside.

ranging from 0.05 mM to 0.2 mM compared with controls. After exposure to SNP for 3 days, ALP activity decreased gradually in the SNP-treated group (Table 1).

In the dexamethasone non-treated group, ALP activity increased only at a SNP concentration of 0.05 mM. ALP activity was lower in SNP in the concentration range from 0.1 to 1.0 mM compared to controls (Table 2).

ALP activity increased at low concentrations of NO but decreased at high NO concentrations. Taken together, these results suggest that low concentration of NO generated by SNP have a stimulatory effect on differentiation of human PDL cells. In addition, SNP appears to have a synergic effect on differentiation of human PDL cells treated with dexamethasone.

Effects of SNP on osteonectin expression

Analysis of protein using western blotting techniques was performed in human PDL cells treated with 0.05 ~ 1.0 mM of SNP for 3, 7 and 14 days. These experiments demonstrated the presence of ON in human

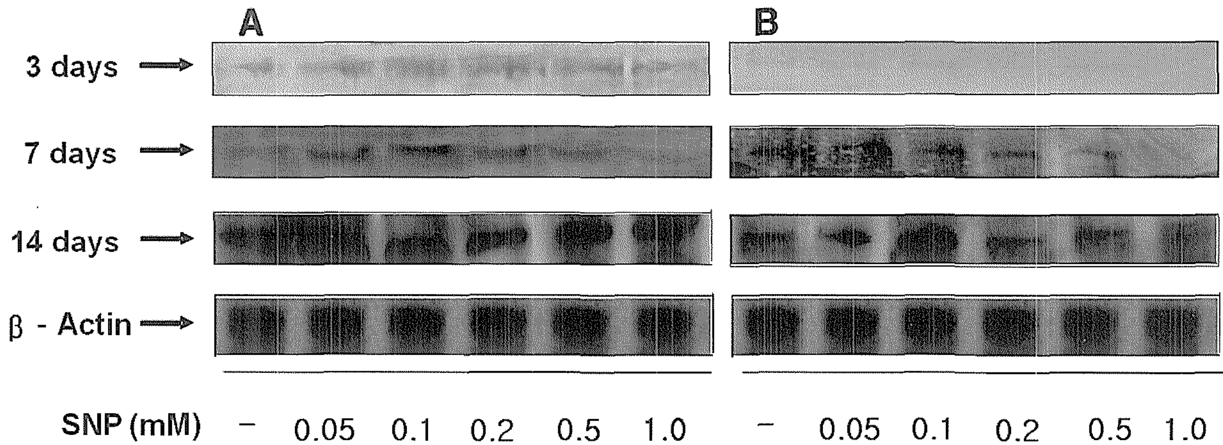

Fig 3. Western blot analysis of BSP expression in human PDL cells cultured with or without dexamethasone and different concentrations of NO for 3, 7, and 14 days. **A,** Expression of BSP in the positive group (with dexamethasone); **B,** Expression of BSP in the negative group (without dexamethasone). Same procedure as designed in the legend to Fig 2. was performed. These data are representative of three independent experiments. *SNP,* sodium nitroprusside.

PDL cells treated with SNP. As shown in Fig 2, ON was very abundant in all the dexamethasone-treated groups after 3 days regardless of the SNP concentration. SNP also induced abundant ON expression in the dexamethasone non-treated group as well as in the dexamethasone treated group compared with controls. However, in the dexamethasone non-treated group, 1.0 mM SNP had less effect on ON expression compared to SNP at concentrations < 0.5 mM. After 7 days, ON expression was decreased with this reduced level remaining after 14 days of SNP treatment.

Effects of SNP on bone sialoprotein expression

The expression of BSP was detected by western blotting analysis in human PDL cells treated with 0.05 ~ 1.0 mM of SNP for 3, 7 and 14 days (Fig 3). BSP expression was not observed in the controls or experimental groups before 14 days. On 14th day, the expression of BSP increased in all the dexamethasone-treated groups regardless of the concentration of NO. In contrast, in cells in the dexamethasone non-treated group, only low concentrations of SNP (especially 0.05 and 0.1 mM SNP) increased BSP expression. BSP expression was not prominent at high concentrations of SNP > 0.2 mM.

DISCUSSION

Identifying the biological properties of human PDL cells will be helpful for understanding the role that these cells play in the various functions of the periodontal ligament, and may improve the success of clinical procedures such as orthodontic tooth movement.[3,17] However, to our knowledge there have been no reports on the effects of NO on human PDL cells. In the present study, we evaluated the effect of NO on cell proliferation and differentiation in human PDL cells.

In our experiments we used SNP as the source of NO, as this compound has the biochemical advantage of permitting investigation of NO's role in signaling transduction pathways without interfering with second messenger systems involving NO.[18] We found that low concentrations of SNP increased cell proliferation, whereas high SNP concentrations (> 0.5 mM) have an inhibitory effect on human PDL cells. This result suggests that proliferation of human PDL cells is dependent on NO concentration. Our results were consistent with similar studies on primary rat osteoblasts,[19] in which low NO concentrations stimulated the proliferation of osteoblasts, whereas high

NO concentrations inhibited cellular proliferation without affecting cellular differentiation.

Alkaline phosphatase activity correlates with bone formation and osteoblast activity in the process of early mineral deposition and calcification.[20] As shown in Table 1, low SNP concentrations induced significantly higher ALP activity in human PDL cells, while high SNP concentrations decreased this activity. This finding of dose-dependent effects of SNP was consistent with the results on cell viability.

Human PDL cells exhibit *in vitro* phenotypic characteristics consistent with osteoblast-like cells.[3] The components of the extracellular matrix (ECM) in bone include collagens and non-collagenous protein, similar to that found in other connective tissues. We examined ON and BSP as markers of osteoblast-like differentiation.[21,22] ON is a major non-collagenous matrix protein and is associated both spatially and temporally with bone cell development. It has been shown to have a variety of roles in the initiation of mineralization in various experimental models.[21] BSP is a major non-collagenous protein in mineralized connective tissue and is considered to be a marker of the late stage of differentiation of osteoblastic cells.[22]

We demonstrated that expression of ON and BSP in human PDL cells was increased by low concentrations of NO, but was decreased by high concentrations of NO. The level of ON was clearly enhanced in cells treated with SNP compared to controls in both the dexamethasone treated and non-treated groups. There was little difference between the dexamethasone-treated groups regardless of the concentration of SNP. However, the expression of ON was decreased in cells treated with 1.0 mM of SNP in the dexamethasone non-treated group. ON was detected mainly in the early stages of culture and with longer culture times the expression of ON decreased in both the control and experimental groups.

Expression of BSP was not detected in the control or any of the experimental groups in the early stages of culture, but during the later stages was expressed more in the SNP-treated human PDL cells than in controls. In the dexamethasone treated group, there was little difference between the experimental groups regardless of the concentration of SNP. In contrast, in the dexamethasone non-treated group BSP expression was greatest at 0.1 mM of SNP and lowest at 1.0 mM of SNP.

As shown by ALP activity and expression of ON and BSP, low concentrations of NO have the potential to stimulate osteoblast-like differentiation of human PDL cells. However, high concentrations of NO have an inhibitory effect. This biphasic effect of NO on human PDL cells is consistent with many other studies.[8,11,12,19] Previous studies reported that NO has biological effects on cells of both osteoclast and osteoblast lineage. It is now well established that high concentrations of NO exert potent inhibitory effects on osteoclastic bone resorption in various *in vitro* model systems including the rat long bone assay,[8] the isolated osteoclast assay[23] and the bone marrow co-culture system.[24] While high concentrations of NO inhibit bone resorption, there is evidence that lower concentrations may be more effective for normal osteoclast function than higher concentrations of NO, and may act by enhancing osteoclast formation and bone resorption.[25]

The effects of NO on bone formation and osteoblast function are consistent with the effects of NO on osteoclasts. High concentrations of NO, generated pharmacologically by NO donors, have potential inhibitory effects on osteoblast growth and differentiation.[25,26] In contrast, low concentrations of NO stimulate proliferation and differentiation of osteoblasts.[27,28]

The range of low concentrations shown to regulate normal osteoblast function is somewhat different between various studies. MacPherson et al. reported that the NO donors, SNP and SNAP (S-nitroso-N-acetylpenicillamine) were able to inhibit cell viability and ALP activity only at high concentrations (> 0.1 mM).[29] Recently, Chen et al. reported that low concentrations of SNP (< 1.0 mM) was not cytotoxic to osteoblasts, but at high concentrations SNP modulated osteoblast metabolism leading to cell death.[30] These differences appear to be dependent on the cell type.

Heme oxygenase (HO) is a rate-limiting enzyme in heme catabolism. The role of heme oxygenase in different tissues has not yet been fully characterized,

but it is becoming evident that it is involved in a variety of cellular regulatory and protective mechanisms.[31] Whether HO-1 is expressed in response to low concentrations of NO, and whether this protein stimulates proliferation and differentiation of human PDL cells requires further research.

Collectively, NO exerts a wide spectrum of effects on human PDL cells, mediating both the physiological and pathological processes. These controversial effects of NO may be explained by the amount of NO produced by activated cells. High concentrations of NO are cytotoxic to human PDL cells in the same manner as in many other different cell types, while low concentrations of NO have been shown to be cytoprotective and to promote cell proliferation and differentiation.

CONCLUSION

The roles of NO in human PDL cells were investigated by performing the MTT assay, and measuring ALP activity and ON and BSP expression using an immunoblotting method. Primary cultures of human PDL cells were treated with NO for two weeks. Low concentrations of NO derived from SNP stimulated proliferation and enhanced ALP activity in human PDL cells. Expression of ON and BSP were prominent when the cells were treated with low concentrations of NO, whereas high concentrations of NO had an inhibitory effect on the expression of ON and BSP.

These findings suggest that NO biphasically modulates proliferation and differentiation in human PDL cells dependent on the exogenous NO concentration. Low concentrations of NO may have a cytoprotective role in human PDL cells.

- 국문초록 -

산화질소가 인간 치주인대세포의 증식과 분화에 미치는 영향

최선영 · 조진형 · 강경화

교정적 치아이동과 밀접한 관련이 있는 치주인대세포에서, 산화질소가 세포의 증식과 분화에 미치는 영향을 알아보고자 하였다. 인간 치주인대세포를 분주한 후에 산화질소의 donor인 SNP의 농도에 따라 실험군을 구분하고, 세포 활성, 염기성 인산분해효소 활성, Western blot 분석을 통한 osteonectin과 bone sialoprotein의 발현 정도를 측정하였다. 0.2 mM 이하의 저농도 SNP를 처리한 실험군에서 대조군과 비교하여 세포 활성, 염기성 인산분해 효소의 활성, 그리고 osteonectin과 bone sialoprotein의 발현이 유의하게 증가하였다. 그러나 1 mM 이상의 고농도 SNP를 처리한 실험군에서는 오히려 감소하였다. 산화질소는 인간 치주인대세포에서 저농도는 세포의 증식과 분화에 촉진 효과를, 고농도는 억제 효과를 보이는 biphasic effect를 갖는다.

주요 단어: 산화질소, 인간 치주인대세포, 증식과 분화

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