

# A study on the mechanism of NO-induced apoptosis in human gingival fibroblast

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## I. Introduction

Nitric oxide (NO) is a short lived, highly reactive free radical gas that is synthesized from L-arginine by the nitric oxide synthase (NOS) of which three isoforms. Periodontal tissues such as gingival fibroblasts and periodontal fibroblasts express at least one of these isoforms; inducible NOS (iNOS) activated by bacterial lipopolysaccharide or cytokines<sup>1,2</sup>. NO acts as an intracellular messenger at physiological level, whereas it can be cytotoxic at high concentration, resulting in cell death such as necrosis and apoptosis<sup>3,4</sup>.

Necrosis is often characterized by cell swelling followed by rupture of the plasma membrane. Apoptosis is characterized by early condensation of nuclear chromatin, cell shrinkage, and DNA fragmentation. Apoptosis is driven from the activation of a family of cysteine protease called caspases, which then cleave a critical set of cellular proteins to initiate apoptotic cell death<sup>5</sup>. These family are expressed as proenzymes and are activated by upstream stimuli.

Among mammalian caspases of at least 14 known members, those involved with apoptosis can be further subdivided into the initiator caspases (-2, -8, -9, and -10) and the effector caspases(-3, -6, and -7)<sup>6,7</sup>.

There are two main pathways activating caspases such as death receptor-mediated and mitochondria-mediated mechanism. Both pathways share the activation of caspase-3 as an executioner caspase, which activates caspase-activated DNase, causing apoptotic DNA fragmentation. Death receptor pathway is stimulated by cell surface death receptors such as tumor necrosis factor(TNF) receptor and Fas<sup>8</sup>. The receptors activated by ligands lead to caspase-8 activation, with subsequent activation of caspase-3. The mitochondrial pathway is initiated from release of cytochrome *c* from mitochondria into cytosol, subsequently resulting in caspase-9 activation which causes caspase-3 activation.

Besides the caspases, members of the Bcl-2 protein family are also critical for the regulation of apoptosis. Bcl-2 family control the release of mitochondrial cytochrome *c* by regulating the permeability of the

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outer mitochondrial membrane. Bcl-2 family members are functionally divided into anti-apoptotic molecules (Bcl-2, Bcl-X<sub>L</sub>, Bcl-W, Mcl-1, A1) and pro-apoptotic molecules (Bax, Bcl-1s, Bid, Bad, Bim, Bik)<sup>6,7</sup>. Among the Bcl-2 protein family, Bcl-2 and Bcl-X<sub>L</sub> are prominent anti-apoptotic family whereas Bax, Bid and Bak are prominent pro-apoptotic family<sup>9</sup>.

There are increasing body of evidences that NO may give rise to cytotoxicity on human gingival fibroblasts because it is recently known that the larger amount of NO is produced by bacterial lipopolysaccharide and cytokines in periodontal tissues<sup>1,10</sup>. However, NO-induced cytotoxicity and its underlying mechanisms have been still not studied in periodontal tissues.

The purpose of this study was to investigate the roles of molecules associated with mitochondria and death receptor-mediated pathway in NO-induced apoptosis of the human gingival fibroblasts.

## II. Materials and methods

### 1. Cell culture and cell viability assay

Human gingival fibroblast (HGF) cells were obtained from healthy gingival tissue of patient in the Department of Periodontics, Chonnam National University Hospital. HGF cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (Gibco, USA) under 5% CO<sub>2</sub> at 37°C. Sodium nitroprusside (SNP, Sigma, USA) was dissolved in distilled DMEM and sterilized through 0.2 µm filter. Cell viability was determined using MTT(3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide, Sigma, USA) assay.

### 2. Nuclear staining with Diff-Quick

Morphological changes of apoptotic cells were

investigated by Diff-Quick stain (Imeb Inc. CA, USA). Cells were plated in 8-well chamber slide at a density of 1x10<sup>5</sup>, incubated for 18 h, and subsequently followed by treatment of 5 mM SNP for 12 h. The cells were then washed with 1x PBS and fixed with acetone and methanol(1:1). After being incubated for 20 min at -20°C, cells were stained with 10 µg/ml PBS containing Diff-Quick stain solution and observed under fluorescence microscope (Olympus, USA).

### 3. Detection of reactive oxygen species (ROS) production and caspase activity

ROS production was monitored by fluorescence spectrometer (Hitachi F-4500, Japan) using 2', 7'-dichlorofluorescein diacetate (DCF-DA). Cells were plated on 96-well plate and treated with N-acetyl-cysteine (NAC, Sigma, USA) and SNP. DCF-DA (25 µM) was added into the media and incubated for further 10 min at 37°C. Emission was measured at 530 nm. Caspase activities were assayed by spectrometer using the caspase-3,-9 activity assay kit (Calbiochem, CA) and caspase-8 activity Kit (Santa Cruz, USA) according to the manufacturer's instructions.

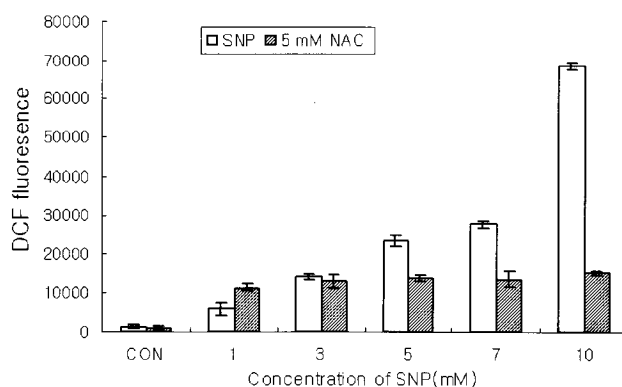
### 4. Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR)

For extraction of total RNA, cells were homogenized in Trizol reagent (Gibco-BRL, USA). RNA samples were quantified by spectrophotometry at 260 nm wavelength. For synthesis of cDNA, 2 µg of total RNA and 2 µl of Oligo-dT (10 pmoles) were mixed with 50 µl RNase-free water, and then incubated at 42°C for 1 h and 94°C for 5 min. PCR products were generated in PCR buffer containing 10 pmoles of each primer using PCR-premix kit (Bioneer, Korea).

After the first denaturation step(5 min at 95°C), samples were subjected to 30 cycles consisting of 40 sec at 95°C, 40 sec at 55°C, and 1 min 30 sec at 72°C, with a final extension step of 10 min, on a GeneAmp PCR system(Perkin-Elmer 2400, USA). The following primer pairs were used: for Bax, 5'-GTT TCA TCC AGG ATC GAG CAG-3'(sense primer) and 5'-CAT CTT CTT CCA GAT GGT GA-3'(antisense primer); for Bcl-2, 5'-CCT GTG GAT GAC TGG TAC C-3'(sense primer), 5'-GAG ACA GCC AGG AGA AAT CA-3'(antisense primer); for Fas, 5'-CAA GGG ACT GAT AGC ATC TTT GAG G-3' (sense primer), 5'-TCC AGA TTC AGG GTC ACA GGT TG-3'(antisense primer). The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator /Polaroid camera System(UVP Laboratories, CA). RT-PCR was performed with primers for the housekeeping gene, GAPDH, as a control. The following primer pairs for GAPDH were used: 5'-TGC ATC CTG CAC CAC CAA CT-3'(sense primer) and 5'-CGC CTG CTT CAC CAC CTT C-3'(antisense primer). The intensity of the obtained bands was determined using the NIH Scion Image Software.

## 5. Western blotting

From cells washed with PBS, proteins were solubilized in the lysis buffer(500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamiden, 1 µg/ml Trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Germany). To determine cytosolic cytochrome *c*<sup>11</sup>, pellet was resuspended in extraction buffer(pH 7.4) containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH, 50 mM KCl, 5 mM EGTA, 2 mM MgCl<sub>2</sub>, and 1 mM DTT. Lysates were incubated for 30 min at 4°C, and centrifuged at 11000xg for 20 min, and protein concentrations were determined by BCA protein assay(Pierce, IL). Protein extracts(100~500 µg) were subjected to electrophoresis on 12% polyacrylamide gel, electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and blocked with 5% skim milk(Becton Dickinson, USA) in Tris-buffered saline-0.1%Tween 20(TBS-T). As primary antibodies, rat monoclonal anti-cytochrome *c*(Pharming, CA), and Bid(Santa Cruz, USA) were used. Blots were subsequently washed in TBS-T for 5 min and incubated with specific peroxidase-cou-



**Figure 1.** ROS production was enhanced in SNP-treated HGF cells. HGF cells loading DCF were incubated for 12 h with SNP alone or co-incubation with 5 mM N-acetyl-L-cysteine (NAC) for 1 h. The intracellular levels of ROS were detected by measuring the DCF-DA fluorescence (Data are mean ± SD from 5 independent experiments.). SNP augmented the production of ROS in a dose-dependent manner and NAC, a free radical scavenger, ameliorated the increments of ROS produced by SNP.

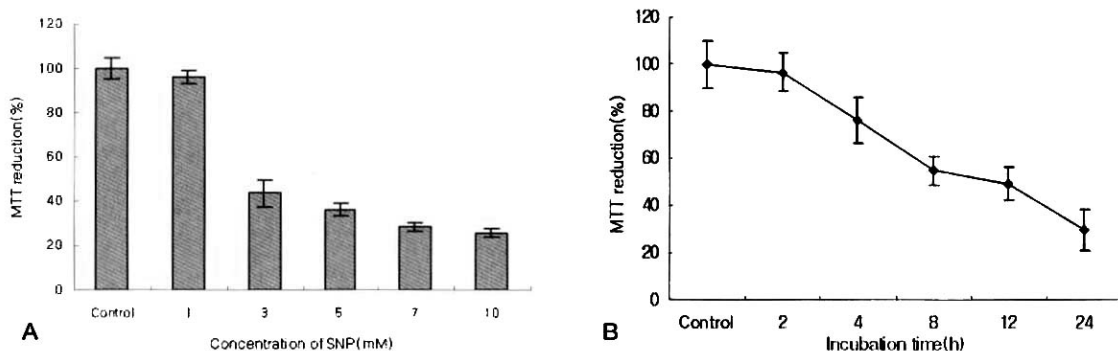
pled secondary antibodies(Sigma, USA). Bound antibodies were visualized using an enhanced chemiluminescent detection system(Amersham Pharmacia Biotech, UK).

### III. Results

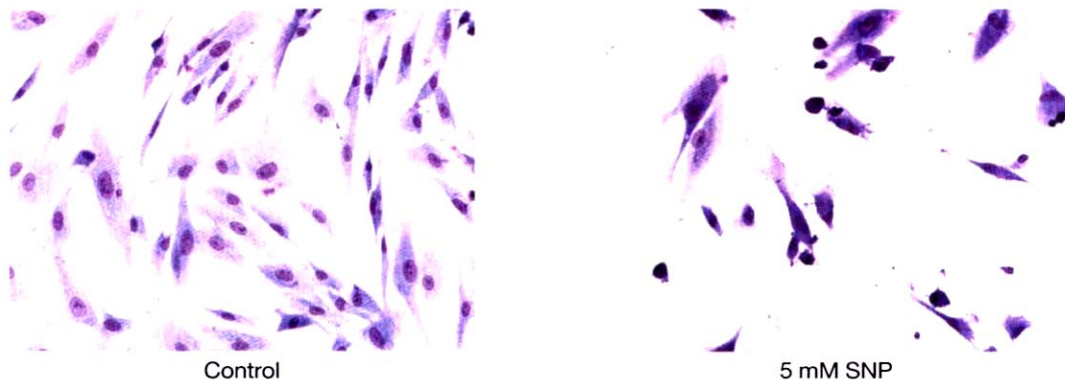
#### 1. NO-induced ROS production and apoptosis in HGF cells

To determine the involvement of ROS in NO-

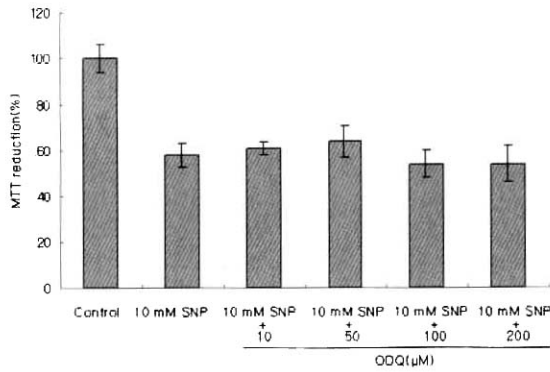
induced cell death of the HGF cells, ROS production was measured using DCF-DA. Figure 1 showed that SNP, a NO donor, enhanced the ROS production in a dose-dependent manner in the HGF cells. Pretreatment of cells with 5 mM NAC, a ROS scavenger, inhibited the increment of ROS produced by SNP. The cell viability determined by MTT assay was gradually reduced in a dose- and time-dependent manner when HGF cells were exposed to SNP(Figure 2A, 2B). The cell survival rate was less than 80% when the cells were treated with 5 mM



**Figure 2. NO induced cell death in HGF cells.** Cell viability was determined by MTT assay. HGF cells were incubated with SNP for indicated concentrations(A) and 5 mM SNP for indicated times (B). Viability of the cells without SNP treatment(Control) was defined as 100%. The viability was reduced in dose- and time-dependent manners in SNP-treated HGF cells (Data are mean  $\pm$  SD from 5 independent experiments).



**Figure 3. The morphologic changes of NO-induced apoptosis in HGF cells.** HGF cells were treated with 5 mM SNP for 12 h and fixed with ethanol and cells were stained using Diff-Quick. Cell shrinkage, chromatin condensation, and DNA fragmentation were shown in SNP-treated HGF cells.



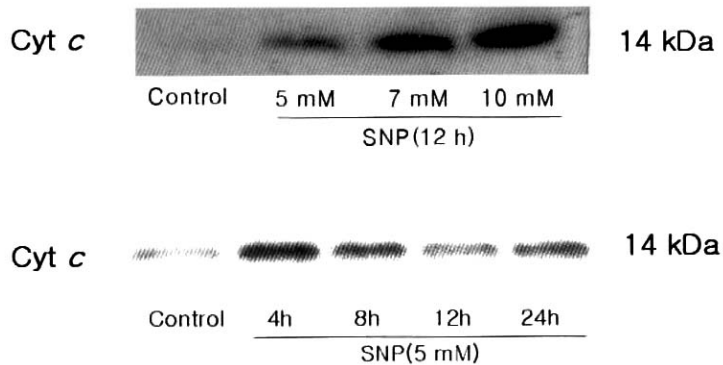
**Figure 4. Cyclic GMP is not involved in NO-induced cell death in HGF cells.** ODQ, a soluble guanylate cyclase inhibitor, did not recover the cell viability decreased by SNP. Results are mean±SD from 5 independent experiments.

SNP for 24 h,

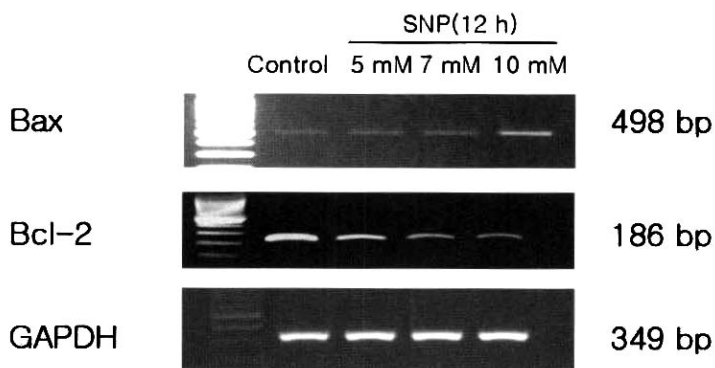
In the presence of 5 mM SNP for 12 h, Diff-Quick staining revealed apoptotic morphological changes, including chromatin condensation and nuclear fragmentation (Figure 3B). Since, NO usually targets soluble guanylate cyclase, effects of soluble guanylate cyclase inhibitor (1H-[1,2,4] oxadiazolo[4,3-a] quinoxaline-1-one, ODQ, 10<sup>-4</sup>M) on NO-induced cell death were examined. ODQ, a guanylate cyclase inhibitor, did not recover the cell viability decreased by SNP (Figure 4), indicating that NO-induced apoptosis is not mediated through cyclic GMP.

## 2. Mitochondria-mediated pathway in NO-induced apoptosis of HGF cells

To evaluate whether mitochondria are involved in NO-induced apoptosis of HGF cells, the amount of cytochrome *c* released from mitochondria into cytosol was determined using cytosolic fractions by western blot after HGF cells were incubated with varying concentrations of SNP for different periods. Cytosolic cytochrome *c* was enhanced in dose-dependent manner in response to exposure of SNP (Figure 5A). Cytochrome *c* showed a peak value at



**Figure 5. Enhancement of cytochrome *c* released from mitochondria into cytosol in SNP-treated HGF cells.** Cytosolic cytochrome *c* was analyzed by immunoblotting with antibody against cytochrome *c*. SNP enhanced the cytochrome *c* released from mitochondria into cytosol in a dose-dependent manner (A) and at 4 h as peak time (B).



**Figure 6. Altered expression of Bax and Bcl-2 in SNP-treated HGF cells.** After incubation of HGF cells with SNP for 12 h, RT-PCR was performed for Bax and Bcl-2 expression, SNP upregulated Bax expression and downregulated Bcl-2 expression in a dose-dependent manner.

4 h of SNP incubation and remained higher than the control values even after 24h of incubation (Figure 5B). This result demonstrates that cytochrome *c* is released from mitochondria into cytoplasm during NO-induced apoptosis in HGF cells.

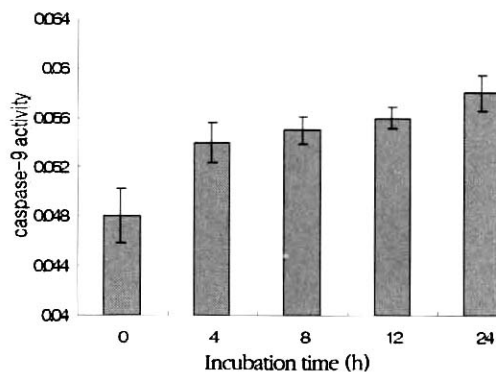
### 3. Bax and Bcl-2 expression in NO-induced apoptosis

Generally, expression ratio of Bax to Bcl-2 has been known to be significant for apoptosis determination, since a high ratio denotes a low apoptotic threshold, whereas a low ratio indicates a higher

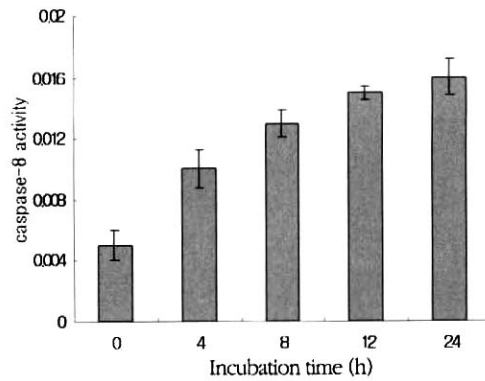
apoptotic threshold. After the treatment of HGF cells with 10 mM SNP for 12 h, the changes in the mRNA level of Bax and Bcl-2 in HGF cells were determined using RT-PCR. Figure 6 showed that SNP upregulated Bcl-2 expression and downregulated the Bax expression in a dose-dependent manner.

### 4. Involvement of caspases in the NO-induced apoptosis in HGF cells

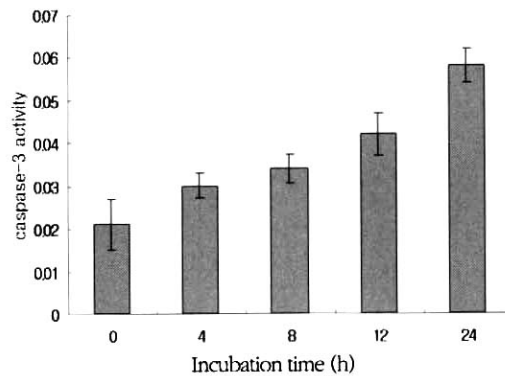
Since it is important to identify the intracellular apoptotic pathways induced by NO in HGF cells,



**Figure 7. Caspase-9 was activated in SNP-treated HGF cells.** Absorbance for caspase-9 activity was measured at 405 nm after incubation with LEHD-pNA substrate(200  $\mu$ M) for 2 h at 37°C. SNP at 5 mM enhanced the caspase-9 activity in a time-dependent manner ( Results are mean  $\pm$ SD from 5 experiments).



**Figure 8. Caspase-8 was activated in SNP-treated HGF cells.** Absorbance for caspase-8 activity was measured at 405 nm after incubation with IETD-pNA substrate(200  $\mu$ M) for 2 h at 37°C. SNP at 5 mM enhanced the caspase-8 activity in a time-dependent manner ( Results are mean $\pm$ SD from 5 experiments.)



**Figure 9. Caspase-3 was activated in SNP-treated HGF cells.** Absorbance for caspase-3 activity was measured in the wells at 405 nm after incubation with DEVD-pNA substrate(200  $\mu$ M) for 24 h at 37°C. SNP at 5 mM enhanced the caspase-3 activity in a time-dependent manner ( Results are mean $\pm$ SD from 5 experiments).

caspases activities were measured on the basis that active caspases consequently cleave their substrate at a specific site. LEHD-pNA (200  $\mu$ M), IETD-pNA (200  $\mu$ M), and DEVD-pNA (200  $\mu$ M) were used as substrates for caspase-9, -8, and -3, respectively. SNP at 5 mM enhanced caspase-8, -9 and -3 activities in a time-dependent manner. It is speculated that both mitochondria and death receptor-dependent apoptotic pathways are involved in NO-induced apoptosis of the HGF cells (Figure 7, 8, 9).

## 5. Fas and Bid expression in NO-induced apoptosis in HGF cells

To know whether death receptor-mediated apoptotic pathway is activated in HGF cells, the mRNA levels of Fas, a death receptor assembly, were determined using RT-PCR. SNP at 5 mM upregulated the expression of Fas in a dose-dependent manner (Figure 10). In addition, Bid level was determined since activated caspase-8 is known to cleave pro-form Bid (pro-Bid) into truncated Bid (tBid). Pro-Bid protein was on the decrease by addition of SNP

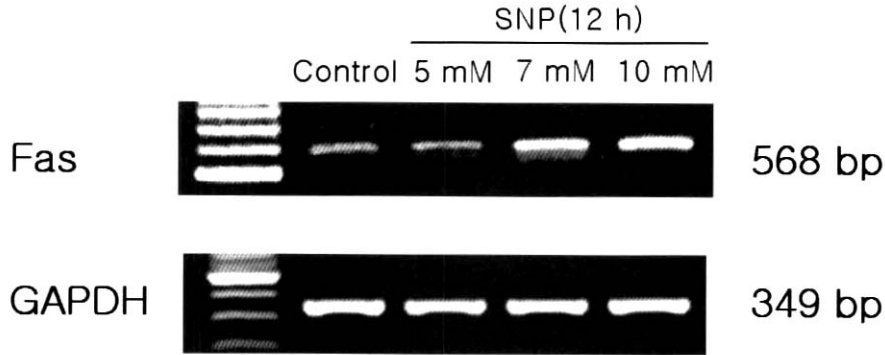


Figure 10. Upregulated expression of Fas in SNP-treated HGF cells, mRNA levels of Fas, one component of death receptor assemblies, was determined by RT-PCR, SNP upregulated Fas expression in a dose-dependent manner.

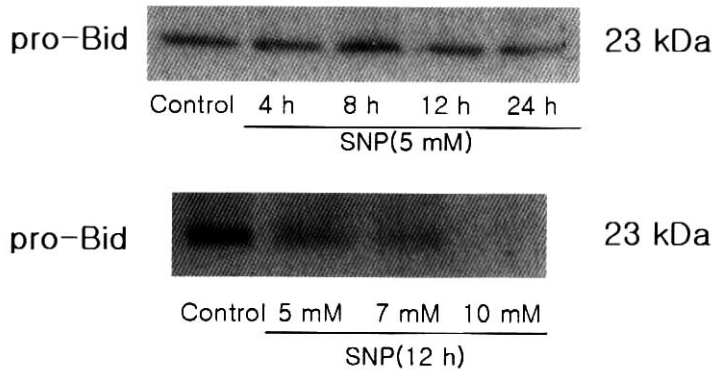


Figure 11. Bid level in SNP-treated HGF cells, SNP reduced the level of the proform Bid (pro-Bid) in a dose- and time-dependent manner.

in a dose- and time-dependent manner (Figure 11). These results suggest that death receptor-mediated pathway plays a crucial role in NO-induced apoptosis of the HGF cells.

#### IV. Discussion

Periodontal tissues such as gingival fibroblast and periodontal fibroblast have a nitric oxide synthases and mass produce NO of high concentration by bacterial lipopolysaccharide and cytokines<sup>1,2</sup>. However there are none of experiments for NO-induced cell death in periodontal tissues,

NO-induced cell death has been classified as

apoptosis and necrosis, on the basis of changes in morphology, enzymatic activity, ATP concentration and adjacent cellular effects<sup>3,4</sup>. The characteristic morphology in apoptotic cell is distinct, including cellular shrinkage, internucleosomal DNA fragmentation and chromatin condensation<sup>12,13</sup>.

In the present study, SNP, a NO donor, decreased the cell viability and gave rise to morphological changes of apoptosis including chromatin condensation, DNA fragmentation, and cell shrinkage in HGF cells. These present results demonstrate the first evidence that nitric oxide induces apoptosis in HGF cells. Previous studies have shown that NO elicit apoptosis through the production of ROS such



as  $\text{H}_2\text{O}_2$  in mitochondria and reaction with superoxide, resulting in formation of peroxynitrite<sup>14,15</sup>. In the present study, SNP enhanced the production of ROS ameliorated by NAC, a free radical scavenger in HGF cells. From these results, it is speculated that NO-induced apoptosis may be in part mediated by ROS in HGF cells, in consistent with those of previous reports in other tissues.

A variety of free radicals such as ROS and peroxynitrite are known to impair mitochondrial function, subsequently resulting in loss of mitochondrial transmembrane potential and release of mitochondrial pro-apoptotic molecules including cytochrome *c*, Smac, apoptosis-inducing factor (AIF)<sup>16,17</sup>. The present study shows that SNP resulted in an increment of cytochrome *c* release from mitochondria into cytoplasm in a dose-dependent manner. Besides SNP enhanced the activity of caspase-9 activated by mitochondrial cytochrome *c*, subsequently resulting in activation of caspase-3 in concert with Apaf-1 and dATP. Taken together, mitochondria-dependent apoptotic pathway is definitely proven to be involved in NO-induced apoptosis of the HGF cells since cytochrome *c* and caspase-9 are major molecules associated with mitochondria-dependent pathway.

In general, caspase-3 is a key and common protease in both mitochondria- and death receptor-dependent pathways and particularly important in free radical-induced apoptosis<sup>18,19</sup>. Previous studies<sup>19,20</sup>, have shown that caspase-3 is activated in response to various ROS. The present study shows that caspase-3 activity was enhanced in SNP-treated HGF cells, which is consistent with that of the previous report in other tissues and cells. From the present study and previous reports, it is assumed that caspase-3 plays a pivotal role in NO-induced apoptosis in HGF cells, even if caspase-independent cell death is proposed to involve in NO-induced cell

death in PC12 cells<sup>14</sup>.

Another possible mechanism for activating caspase-3 is caspase-8 mediated process activated by Fas and TNF receptor-1. Recent studies have reported that ROS such as  $\text{H}_2\text{O}_2$  directly induces upregulation of death receptor assembly such as Fas and Fas ligand, subsequently activating caspase-8<sup>16,21</sup>. From these previous reports, it is proposed a possibility that death receptor-dependent apoptosis pathway may be involved in caspase-3 activation in NO-induced apoptosis of HGF cells. In the present study, Fas, a death receptor assembly, was upregulated and caspase-8 activity was enhanced in SNP-treated HGF cells. The present study shows the first evidence that death receptor-dependent pathway may be involved in NO-induced apoptosis of HGF cells. From the present results, NO-induced apoptosis is likely to be mediated by both mitochondria and death receptor-mediated pathways in HGF cells.

On the otherhand, the Bcl-2 family of proteins are known to be well-characterized regulators of cytochrome *c* release from mitochondria into cytosol. The Bcl-2 subfamily contains anti-apoptotic proteins such as Bcl-2 and Bcl-X<sub>L</sub>, which reduce cytochrome *c* release and a loss of mitochondrial transmembrane potential ( $\Delta\psi_m$ )<sup>22,23</sup>. The Bax subfamily contains pro-apoptotic proteins such as Bax and Bak, which induce cytochrome *c* release and a loss of  $\Delta\psi_m$ <sup>24</sup>. Bcl-2 proteins such as Bid, Bik and Bim are another subfamily of pro-apoptotic proteins, which are activated by caspase-8. Thus, ratio of pro-apoptotic and anti-apoptotic caspases may be pivotal cue to release of cytochrome *c* from mitochondria into cytosol. Therefore, expression of Bcl-2 family was examined in the present study to elucidate the involvement of Bcl-2 family in NO-induced apoptosis. In the present study, Bcl-2 mRNA was downregulated, whereas Bax mRNA was upregulated in SNP-treated HGF cells. Even in previous

reports<sup>25,26</sup>, NO has been reported to directly or indirectly regulate Bcl-2 family expression in other tissue and cells. These present results suggest that Bcl-2 proteins are involved in NO-induced apoptosis of HGF cells. An interesting result is that Bid was activated by SNP in the present study since Bid is known to be activated by caspase-8, unlike other Bcl-2 family. From the present data, it is speculated that there is an interrelation lineage between the death receptor-mediated apoptotic signals and the mitochondria-mediated apoptotic signals. However, roles of Bcl-2 family may be a debate in NO-induced apoptosis of HGF cells since Bcl-2 family regulates the production of ROS and cytochrome c release from mitochondria into cytosol<sup>22,24</sup> and ROS could conversely regulates the expression of Bcl-X<sub>i</sub> mRNA<sup>17</sup>. Further researches are required to determine the roles of the Bcl-2 family in NO-induced apoptosis of HGF cells.

In general, NO acts a variety of physiological function through activating soluble guanylate cyclase, subsequently resulting in synthesis of cyclic GMP. Thus, it was examined whether cyclic GMP pathway might be involved in NO-induced apoptosis of HGF cells. In the present study, ODQ, a soluble guanylate cyclase inhibitor, did not ameliorated the cell viability reduced by SNP in HGF cells. This result suggests that NO-induced apoptosis is not mediated by cyclic GMP pathway. In summary, the results from this study suggest that NO induces apoptosis through activation of both the mitochondria- and death receptor-dependent pathways mediated by ROS and Bcl-2 family in HGF cells.

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## 사람 치은 섬유아세포에서 산화질소 유도 세포고사에 대한 연구

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산화질소는 생리적 농도에서 세포내 신호전달자로 작용하지만 높은 농도에서는 세포독성을 일으킨다. 최근 치은 섬유아세포와 치주인대 섬유아세포는 산화질소 합성효소를 가지고 있고 세균의 lipopolysaccharide나 cytokine에 의해 대량의 높은 농도의 산화질소가 합성된다는 보고가 있음에도 지금까지 치은 조직에서 산화질소의 세포독성에 대한 연구는 아직 이루어 지지않고 있다. 본 연구는 사람의 치은 섬유아세포에서, 산화질소유도세포 고사기전을 밝히는데 목적이 있다.

세포 성장력은 MTT 방법으로 측정하였고, 세포의 형태적 변화는 Diff-Quick 염색법으로 조사하였다. Bcl-2 family와 Fas 발현 정도는 RT-PCR 방법에 의해 확인하였으며, caspase-3, -8 와 -9의 활성은 spectrophotometer 로 reactive oxygen species (ROS)는 형광분광계에 의해 측정되었다. 미토콘드리아에서 세포질로 분비된 cytochrome c는 western blot으로 조사하였다.

산화질소 유리제인 sodium nitroprusside (SNP) 처리는 사람 섬유아세포의 생존률을 시간과 농도 의존적으로 감소시켰고, 세포용적축소, 염색사 응축, DNA 절편화를 일으켰다. 또한, SNP 처리로 미토콘드리아에서 세포질로 유리되는 cytochrome c 양이 증가되었고, caspase-9 과 caspase-3 의 활성이 증가되었다. 한편, SNP 처리에 의해 death receptor 구성요소인 Fas 발현이 증가되었고, caspase-8의 활성이 증가되었다. Bcl-2 family 에 대한 RT-PCR 분석결과, 세포고사를 억제하는 Bcl-2 발현은 감소되었으나 세포고사를 자극하는 Bax와 Bid의 발현은 증가되었다. Soluble guanylate cyclase 억제제인 ODQ는 SNP에 의한 세포 생존을 감소를 차단하지 못했다. 따라서, 본 실험의 결과들은 사람 섬유아세포에서 산화질소유도 세포고사에 Bcl-2 family나 ROS가 매개하는 미토콘드리아 의존 및 death receptor 의존 세포고사기전이 관여함을 시사하였다.

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주요어: 치은 섬유아세포, 산화질소, 세포고사, 신호경로