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Effect of aging on expression of nitric oxide and inducible nitric oxide synthase in human gingival fibroblasts

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I. Introduction

In general, aging has been defined as a progressive decrease in the physiological capacity and the reduced ability to respond to environmental stresses lead to an increased susceptibility to disease[1]. It is well known that the severity of periodontal diseases is affected by the host's age[2]. In addition, recent studies have shown that the production of inflammatory mediators such as PGE2, IL-1β, IL-6, cyclooxygenase-2 and the plasminogen activator are higher in the gingival fibroblasts from the older animals or in vitro senescent fibroblasts compared with younger counterparts[3-7]. Although the severity of periodontal disease is affected by age, functional changes in the periodontal tissue cells during the aging process have not been well characterized.

Recent studies have shown that activation of the iNOS pathway is essential for IL-1-stimulated bone resorption, both in vivo and in vitro[8,9]. Although the precise role of NO in the immunopathological process is unknown, the result of this process is the destruction and ultimately the loss of both the soft and hard tissues surrounding the tooth[10].

Immunohistological studies of human iNOS in gingival tissue have shown that iNOS is widely distributed in epithelial cells, endothelial cells, fibroblasts, macrophages, and polymorphonuclear leukocytes[11]. In addition, it has recently been demonstrated that synthesized NO and NO production was increased by stimulating the cultured human gingival fibroblasts

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with bacterial lipopolysaccharide (LPS) and IFN-γ[12].

Hayflick's theory suggests that the number of cell divisions prior to the onset of senescence is inversely correlated with the age of the tissue donors and that cellular aging in vitro reflects aging in vivo[13]. This has been accepted for examined the process of cellular aging in fibroblasts, In addition, the in vitro use of a method that involves increasing population doubling is a useful model for a cellular-aging study.

As the major cell type in the periodontal connective tissue, gingival fibroblasts are considered to be important cellular components in periodontal disease[14]. In the inflammatory periodontium, gingival fibroblasts contribute to the pathogenesis of periodontal disease by the increased secretion of inflammatory mediators[12]. Moreover, NO exists for approximately 6-10 sec and is then converted by into NO_2^- and NO_3^- by O_2 and H_2O[15]. Because NO with its short half-life may not diffuse far from the site of NO production in the inflamed tissue[16], it shows that the gingival fibroblasts can produce NO.

Porphyromonas gingivalis (P. gingivalis) is a Gram-negative, anaerobic rod that is considered to be one of the main bacteria associated with adult periodontitis[17], and its LPS in the cell wall is believed to be one of the virulent factors associated with the development of periodontitis[18]. IFN-γ acts synergistically with LPS to induce the secretion of NO by activating a number of transcription factor signaling cascades[19].

From this background, this study examined the effect of the cellular aging on iNOS and NO production in human gingival fibroblasts. This study proposes a hypothesis to explain the age-associated alterations in the cells comprising the periodontal tissues as well as their response to stimuli such as a bacterial invasion and inflammatory mediators.

II. Materials and Methods

1. Cell Culture

Human gingival fibroblasts were prepared according to the method reported by Somerman et al.[20]. Young cells were defined as those with 4 population doublings of hGFs from a 10-year-old patient (young hGFs). Old cells were defined as 4 population doublings of hGFs from a 55-year-old patient (old hGFs) and those with 15 population doublings of hGFs from a 10-year-old patient (P15 hGFs). Informed consent was obtained from all the patients after the nature of the study had been fully explained.

2. Growth of Bacteria, Preparation of Lipopoly-saccharide and Sodium Dodecyl Sulfate Polyacrylamide Gel Electropho resis (SDS-PAGE)

The bacterial strain used in this study was P. gingivalis ATCC 33277. The strain was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). P. gingivalis was cultured in Tryptic Soy broth supplemented with 0.5% yeast extract, 0.05% cysteine HCl-H2O, 0.5 mg ml-1 of hemin and 2 g/ml of vitamin K1. The strain was grown at 37℃ in an anaerobic chamber under 10% H2, 10% CO2 and 80% N2. The LPS was extracted using the hot phenol-water method reported by Westphal et al[21]. LPS derived from Escherichia. coli Serotype 0111:B4 (Sigma, Inc.,

St. Louis, MO, USA) was used as a reference for the biological assay and for SDS-PAGE. The endotoxin was analyzed by SDS-PAGE as described by Diedrich et al[22]. The endotoxin was detected in the gel by silver staining[23].

3. Fibroblast viability (MTT) Assays & Challenge of hGFs with LPS and IFN-γ

The cell viability was determined by the ability of the cells to metabolically reduce the tetrazolium salt, 3-[4,5-demethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide(MTT), to a purple formazan dye. The absorbance was read at 565 nm using a microtiter plate spectrophotometer, and results were expressed as %control.

4. Nitrate and nitrite estimation

NO production was assayed by quantifying the stable NO metabolites, nitrite and nitrate, in a microplate adaptation of the Griess assay, as previously described by Nagano[24]. NO production was determined on a cell-free supernatant by the measuring the NO_2 concentration using a spectrophotometric assay based on the Griess reagent (1% sulfanilamide, 0.1% naphtylendiamine dihydrochloride, 2% H3PO4) and incubating at room temperature for 10min. The absorbance at 550 nm was measured spectrophotometrically. The NO2 concentration was calculated from a standard curve and is expressed as μM/105 cells.

5. Immunohistochemical and Immunocytochemical Staining for iNOS protein

A standard immunostaining procedure was used to detect the iNOS in the human gingival tissue and cultured human gingival fibroblasts. The negative controls included the omission of the primary antibody, in the presence of all the other steps.

6. RT-PCR for iNOS mRNA

The oligonucleotide PCR primer specific for iNOS and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were synthesized by Bioneer (Bioneer, Korea). The primers for iNOS (490bp) were: 5'-ATG GAA CAT CCC AAA TAC GA-3' and (antisense) 5'-GTC GTA GAG GAC CAC TTT GT-3',(as described by Yanagita)(Yanagita et al. 2002). while those for GAPDH (200bp) were: 5'-CCA TGG AGA AGG CTG GG-3'and (antisense) 5'CAA AGT TGT CAT GGA TGA CC-3'. The HGFs were cultured with P. gingivalis LPS (5 μg/ml) and/or IFN-γ (20 ng/ml), harvested and then washed throughly with PBS. The total RNA was prepared by homogenization in TRIzol Reagent (Invitrogen, Carlsbad, CA). All the reactions were subjected to different amplification cycles using a programmed thermal cycler (Perkin-Elmer Cetus) under the following conditions: iNOS mRNA: 94℃ for 1 min, 43℃ for 1 min, and 72℃ for 1 min and GAPDH mRNA: 94℃ for 1 min, 52℃ for 1 min, and 72℃ for 1 min. The PCR fragments were electrophoresed on 1.5% agarose-ethidium bromide gels run at 100V for 30 min. The relative intensities from the most typical cycle were quantified by densitometry and normalized to GAPDH.

7. Statistical Analysis

Statistical analysis was performed using SPSS. A one-way ANOVA was used for all the analyses with the power of the performed tests at $\alpha=0.05$. A Tukey-HDS test was used spe-

Table 1. Survival rates of hGFs treated with P. gingivalis LPS.

Concentration of LPS (μg/ml)	M±SD perwell (nm)	Survival rate compared with control (%)
0(control)	0.24±0.0035	100
2.5	0.23±0.0175	95
5	0.23±0.0135	94
25	0.19±0.0015	80
50	0.18±0.041	74
100	0.17±0.008	71

M±SD: Mean ± Standard

cifically for the pairwise comparisons based on the studentized range. A t-test was used to compare the positive control, only media group and experimental groups used T-test (< 0.05)

III. Results

The microscopic observation, showed that the young hGFs were more packed and spindle-like shaped, and the old hGFs were slightly larger than the young cells (Figure 1).

In the MTT assays, the LPS from P. gingivalis caused a dose-dependent suppression of the growing young hGFs. Inhibition in this single experiment ranged from 4% at 2.5 μg/ml to 29% at 100 μg/ml (Table 1).

P. gingivalis LPS exhibits a series of distinct bands corresponding to a microheterogeneity of the molecular sizes within the extract (Figure 2).

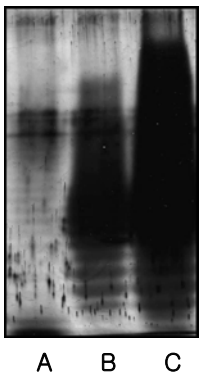

Figure 2. SDS-PAGE of (A) E. coli LPS (1μg); (B) P. gingivalis LPS (1x); (C) P. gingivalis LPS (5x). The amount of P. gingivalis LPS was quantitated by the relative intensities from E. coli LPS (1 μg) used as a reference.

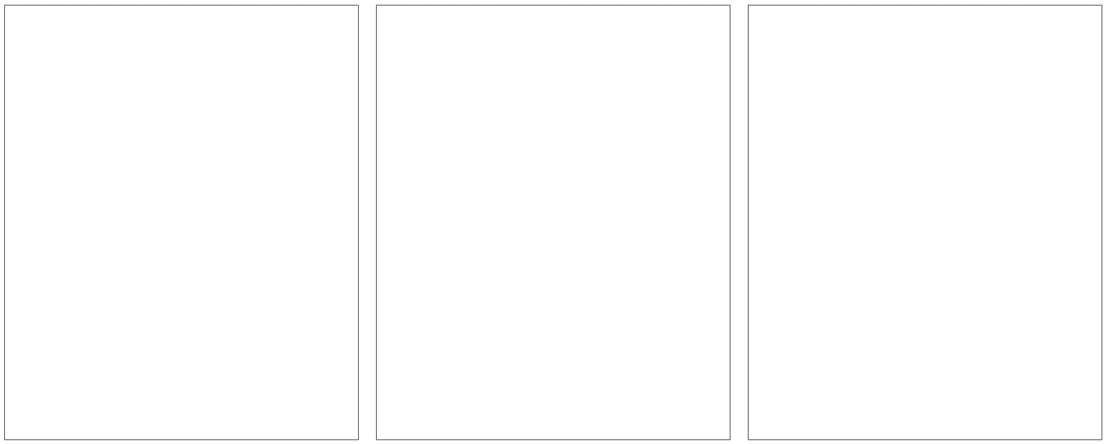

Figure 1. Phase contrast micrographs of hGFs. (A) Young hGFs; (B) Old hGFs; (C) In vitro senescence P15 hGFs.

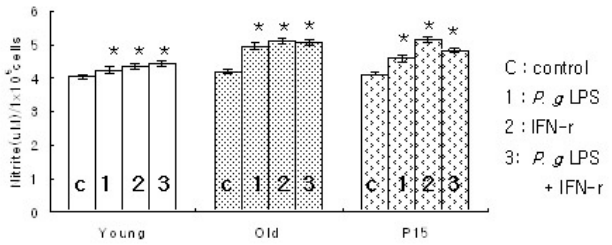

Figure 3. Comparison of NO production between control and experimental groups in the young, old and P15 hGFs. NO production in the presence of P. g LPS and/or IFN-γ in young, old and P15 hGFs was significantly higher than in corresponding control.
* : significantly different NO production between the control and the experimental groups. P<0.05

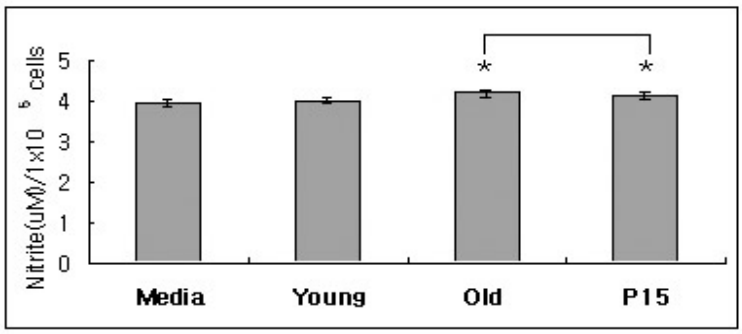

Figure 4. Comparison of NO concentration between only media and unstimulated young, old and P15 hGFs. The NO production in the absence of LPS and IFN-γ in young GFs did not differ significantly from media. The NO concentration in old and P15 hGFs was significantly increased than that of media alone. The NO concentration of old hGFs was not different from that of P15 hGFs.
* : significantly different NO production between the media and basal levels of young, old and P15. P<0.05

This study determined the basal and LPS and/or IFN-γ-stimulated level of nitrate/nitrite. NO production in the presence of LPS and/or IFN-γ in young, old and P15 hGFs was significantly higher than that in the corresponding control (Figure 3). The NO concentration in the old and P15 hGFs was significantly higher than that in the media alone (Figure 4). LPS and/or IFN-γ-stimulated NO production in the old hGFs was significantly higher than that in the young hGFs (Figure 5,6,7). The level of NO production in the cells from young, old and P15 hGFs tended to increase with the incubation period over 24 h (Figure 8, Table 2).

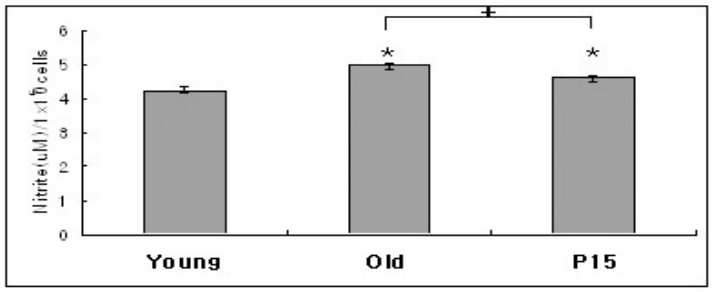

Figure 5. P. gingivalis LPS-stimulated NO production in the young, old and P15 hGFs.
P. gingivalis LPS-stimulated NO production in the old and P15 was significantly higher than that in the young hGFs. The amount in the old hGFs was higher than that in the P15 hGFs.
* : Significantly different from corresponding young cells with P. gingivalis LPS. P<0.05
+ : sinificantly higher NO production in the P. gingivalis-stimulated old hGFs compared with P15 hGFs. P<0.05

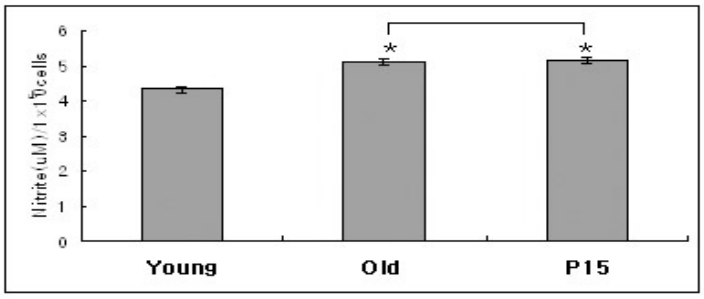

Figure 6. IFN-γ-stimulated NO production in the young, old and P15 hGFs.
IFN-γ-stimulated NO production in the old and P15 was significantly higher than that in the young hGFs. The amount in the old hGFs was similar to that in the P15 hGFs.
* : Significantly different from corresponding young cells with IFN-γ. P<0.05

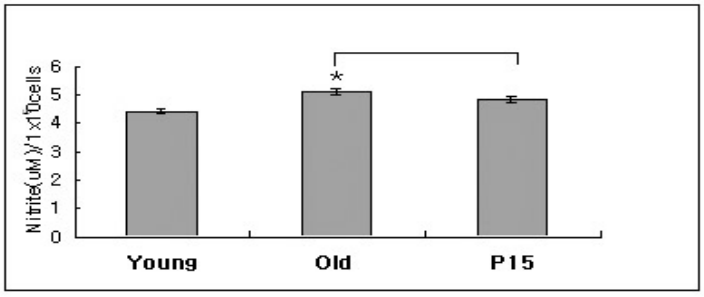

Figure 7. LPS and IFN-γ-stimulated NO production in the young, old and P15 hGFs.
LPS and IFN-γ-stimulated NO production in the old was significantly higher than that in the young hGFs. The amount in the P15 hGFs was not different from the that of young GFs but similar to that in the old hGFs.
* : Significantly different from corresponding young cells with P. g LPS + IFN-γ. P<0.05

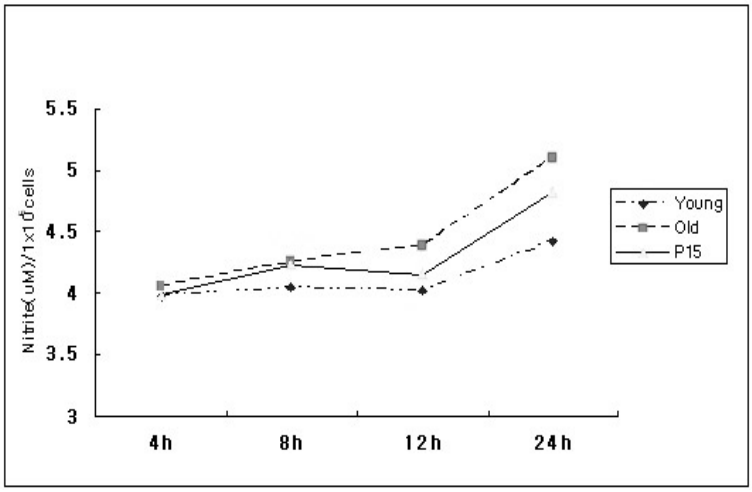

Figure 8. Effect of incubation time on NO production in the young, old and P15 hGFs. The amount of NO in the old hGFs significantly different from the young hGFs at 8 and 24 h. There were not significant differences between the old hGFs and the P15 hGFs.

Table 2. Nitrite production in the young, old and P15 hGFs according to incubation time.

(μM/1x10^5 cells)

Incubation Time (h)	Young	Old	P15
4h	3.98±0.01*	4.06±0.06	3.99±0.02
8h	4.05±0.01	4.26±0.09†	4.23±0.01
12h	4.02±0.02	4.38±0.18	4.15±0.05
24h	4.42±0.09	5.10±0.16†	4.82±0.15

*Mean ± SD. †Significantly different from corresponding young cells with P. g LPS + IFN-γ, P<0.05

The LPS and IFN-γ-stimulated NO production in the old hGFs was higher than that in the young hGFs at 8 and 24 h. When the amounts of these factors produced by the old and P15 hGFs were compared to those produced by the young hGFs (Table 3), These amounts

Table 3. Nitrite production in the young, old and P15 hGFs stimulated LPS and/or IFN-γ.

(μM/1x10^5 cells)

	Young	Old	P15	Rate Old/Young (fold)	Rate P15/Young (fold)
Control	4.02±0.03*	4.21±0.07‡	4.14±0.01	1.05	1.03
P. g LPS	4.25±0.05†	4.50±0.10†‡	4.60±0.10†‡	1.06	1.08
IFN-γ	4.36±0.04†	5.12±0.12†‡	5.20±0.10†‡	1.17	1.19
P. g LPS+IFN-γ	4.42±0.09†	5.10±0.16†‡	4.82±0.15†	1.15	1.09

*Mean ± SD. † Significantly different from corresponding control, P<0.05

‡Significantly different from corresponding young cells, P<0.05

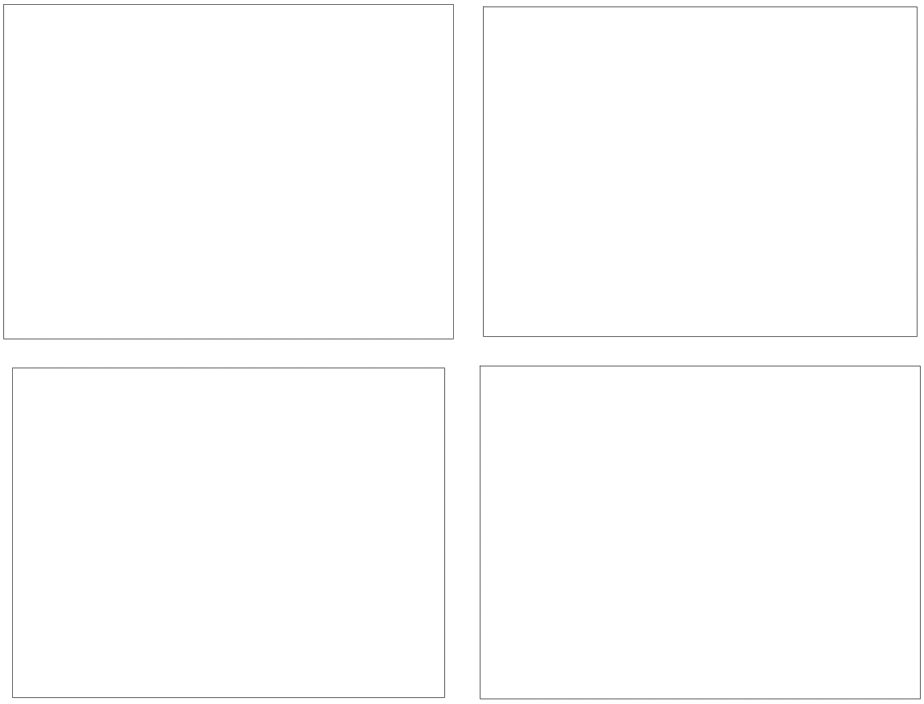

Figure 9. Expression of NOS-II in young(20-year-old) and old(55-year-old) human gingival tissue(200x magnification).
A : NOS-II immunostaining of healthy young gingival tissue.
Note expression in basal keratinocytes and gingival fibroblasts.
B : NOS-II immunostaining of healthy old gingival tissue.
Note expression in basal keratinocytes and gingival fibroblasts.
C : NOS-II immunostaining of inflamed young gingival tissue. Note the stronger expression in keratinocytes than that in healthy young gingival tissue. Some of inflammatory cells were strongly stained.
D : NOS-II immunostaining of inflamed old gingival tissue.
Note expression in endothelial cells and inflammatory cells around small vesseles.
→ : Basal keratinocytes, ▲ : Gingival fibroblasts
* : Inflammatory cells, △ : Endothelial cells

were higher in the LPS and IFN-γ-stimulated groups than in the control groups.

The inflamed gingival tissue exhibited very heavy immunostaining for NOS-II, with strong staining of the fibroblasts and inflammatory cells (Figure 9). The cytoplasm of the aged hGFs was more hypertrophied than the young cellular cytoplasm and included a large number of vacuoles inside and showed strong staining to NOS-II.

The staining of NOS-II in the cultured HGFs was observed mainly in the cytoplasm. The cytoplasm of the aged HGFs was more hypertrophied than the young cellular cytoplasm and it included numerous vacuoles inside and showed strong staining to NOS-II (Figure 10-C,D).

RT-PCR analysis revealed that the level of unstimulated iNOS mRNA in the old and P15 hGFs was higher than that in the young hGFs

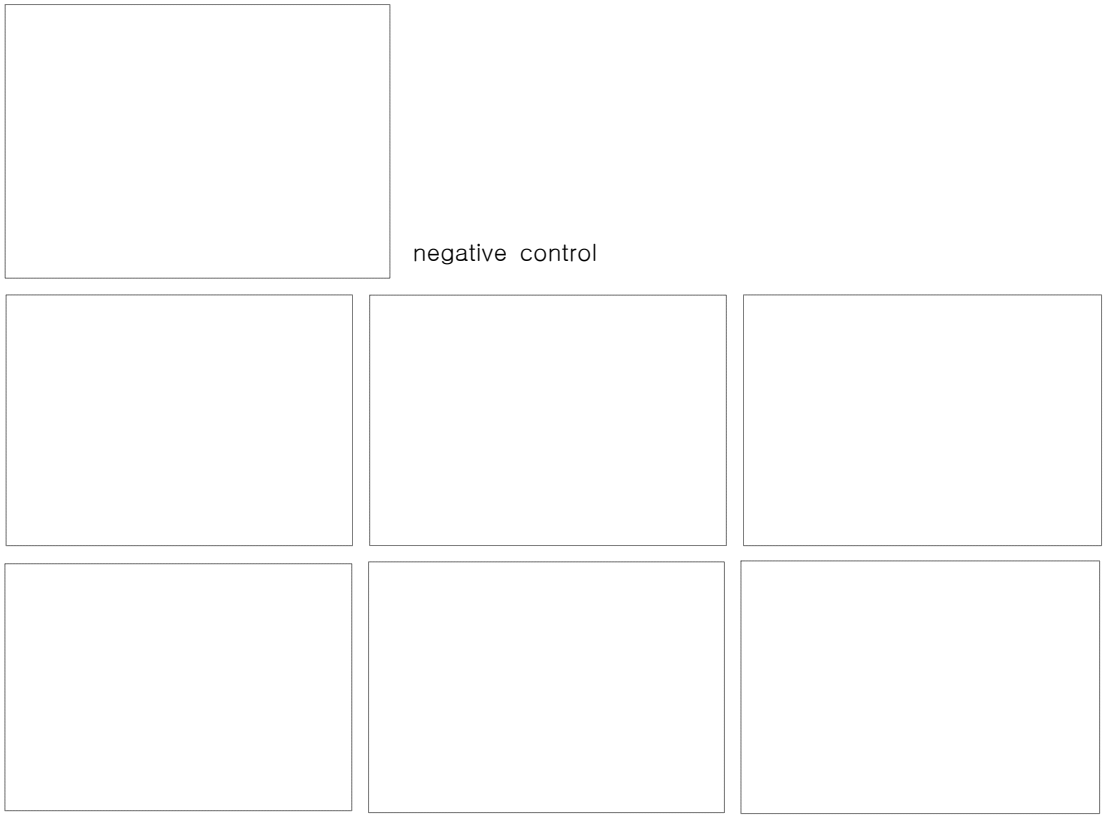

Figure 10. Immunostaining of NOS-II in the cultured young, old and P15 hGFs (400 x magnification).
Negative control: Specimen stained in the absence of primary antibody.
A : Unstimulated young hGFs. B: LPS(P. gingivalis, 5 μg/ml) and IFN-γ (20 ng/ml)-stimulated young hGFs. C: Unstimulated old hGFs. D: LPS and IFN-γ-stimulated old hGFs. E: Unstimulated P15 hGFs. F: LPS and IFN-γ-stimulated P15 hGFs.

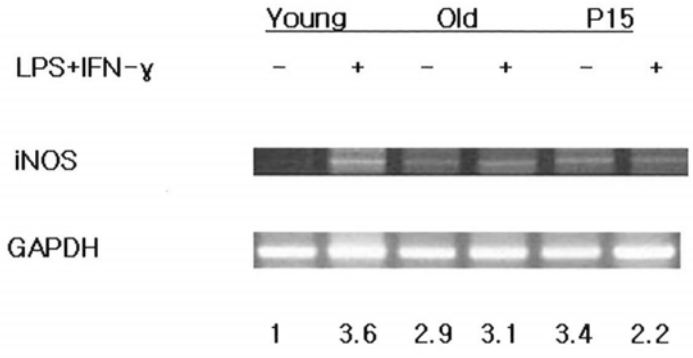

Figure 11. RT-PCR analysis of the iNOS mRNA expression level. The mRNA of iNOS (upper panel) and GAPDH (lower panel) are shown. The numbers below indicate the iNOS to GAPDH ratio when the density of the non-stimulated young was graded at 1.

(Figure 11). After 24 h incubation with the LPS and IFN-γ, the iNOS mRNA of the young hGFs appeared to be approximately 3.6-times higher than the basal level.

IV. Discussion

A large majority of studies examining the effect of aging on periodontal disease used in vitro aged gingival fibroblasts according to a method of increasing population doubling as an aging model[4-6]. Although cultured diploid fibroblasts have played an increasingly important role in experimental studies of aging at the cellular level[25], it has been demonstrated that only certain phenotypes survive during subcultivation[5]. Therefore, some studies used the in vivo cellular aging experimental model, the cells from old rats (20 months old) or Down's syndrome patients who showed premature aging[3,7]. Okamura et al[3]. demonstrated that the LPS-stimulated PGE2 and IL-1β production in aging GFs in vitro were much higher than that in the young GFs. These results were later confirmed by cultured GFs from the gingiva removed from old rats (20 months old)[3]. This suggested that in vivo aging using an animal is also a useful model for a longitudinal study examining age as a risk factor of periodontitis. In this study, the old gingival fibroblasts were cultured from the gingiva of an older (55-year-old) patient, as an in vivo cellular aging experimental model. This type of study examining the effect of aging on periodontal disease was first attempted using the cells from an older humans. The in vitro senescence cells (Passage 15 of the young GFs) were also used as an aging model in order to confirm the results from the in vivo cellular aging model. In this study, the morphological characteristics of the old hGFs was similar to the P15 hGFs, and the features of NO production from both the old and P15 hGFs was similar to each other. It is interesting that the non-stimulated NO production by the older and P15 hGFs was higher than that by the younger hGFs.

In this study, the expression levels of factors related to periodontal disease, such as PGE2, IL-1β, IL-6, cyclooxygenase-2 and the plasminogen activator were similar regardless of age in the absence of stimulation[3-6], while the level of these factors were markedly higher in the old cells than in the old cells than in the young cells in response to exogenous stimuli. However, significant NO hypersecretion was observed in the older and P15 hGFs compared with those obtained from the young patients in absence of stimulation. Unlike the other factors related to periodontal disease, NO is a free radical. Free radicals, which are byproducts of normal cellular oxidative processes[26], have been shown to be involved in senescence[27]. Senescent cells have higher levels of free radicals than normal cells[28]. In addition, Richmonds et al.[29] reported that NO was related to aging. Although the mechanisms for the age-associated increase in NO expression are unclear, oxidative damaged mitochondria is suggested to be a major factor of the age-associated increase in the free radical concentration.

While the eNOS and nNOS synthesize NO at low levels (picomolar range) constitutively, iNOS is expressed upon stimulation by proinflammatory mediators, such as IL-1β, TNF-α and IFN-γ at high levels (nanomolar range)[30].

s. 5. RT-PCR analysis revealed that the iNOS mRNA level in the old and P15 hGFs in the absence LPS and IFN- γ was higher than that in the younger hGFs. This suggests that the aging of hGFs plays an important role in

the progression of periodontal disease by affecting the producing of NO and iNOS production in the basal level and in response to both LPS and IFN-γ. Furthermore, considering the treatment of periodontal disease from the host side, the inhibition of the inflammatory responses induced by exogenous stimuli may be one powerful approach for reducing the level of tissue degradation. Therefore, in order to minimize the susceptibility to periodontal disease and reduce the exposure of exogenous stimuli such as LPS from plaque in the aged population, it may be necessary to maintain healthy periodontal tissue

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-Abstract-

노화가 사람 치은섬유아세포의 nitric oxide와 inducible nitric oxide synthase 발현에 끼치는 영향

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치주질환의 진행이 나이에 의해 영향을 받는다는 사실은 알려져 있으나 노화에 따른 치주조직 세포의 기능적인 변화에 관한 사실은 많이 알려져 있지 않다. 노화에 따른 세포의 노화가 치주질환의 진행에 어떠한 영향을 끼치는가를 아는 것은 중요하다. 염증 상태에서 nitric oxide (NO)는 조직 파괴에 관여하는 인자로 작용하여 치주질환의 진행에 관여하는 것으로 알려져 있다. 따라서 이 연구는 사람의 치은에서 배양된 치은섬유아세포를 이용하여 세포의 노화에 따른 NO와 이의 합성효소인 inducible nitric oxide synthase (iNOS)의 발현을 알아봄으로써 세포의 노화가 치주질환의 진행에 끼치는 영향에 대해 알아보고자 하였다.

10세의 환자와 55세의 환자에서 각각 채취한 치은에서 배양된 세포와 10세의 환자에서 채취한 세포를 계속적인 계대배양을 통해 얻은 실험실 상 노화된 세포를 포함하여 총 3 종류의 치은섬유아세포를 실험에 이용하였다. Hot phenol-water extraction을 통해 추출된 Porphyromonas. gingivalis ATCC 33277 lipopolysaccharide (LPS)와 재조합 IFN-γ를 세포에 적용시켜 Griess assay를 통해 조건화된 배지에서 NO를 측정하였다. 20세와 55세의 환자에서 채취된 치은 조직과 총 3 종류의 배양된 세포에 NOS-II 항체를 적용시켜 iNOS 단백질 발현을 관찰하였다. Total RNA를 추출하여 RT-PCR를 통해 iNOS mRNA의 발현을 분석하였다.

치은섬유아세포에서 NO는 자발적으로 발생되었고, 이러한 발현은 젊은 세포보다 노화된 세포에서 강하였다. P. gingivalis LPS와 재조합 IFN-γ는 치은섬유아세포에서 NO의 발현을 증가시켰고, 이러한 발현은 젊은 세포보다 노화된 세포에서 강하였다. 면역조직화학 염색에서 iNOS 단백질은 젊은 사람과 노화된 사람의 치은 조직 모두에서 치은섬유아세포와 상피의 기저층 세포와 염증세포에서 발현되었으나 노화에 따른 발현의 차이를 구별할 수는 없었다. 세포의 면역염색에서 iNOS 단백질은 노화된 세포에서 강하게 발현되었고 이러한 발현은 LPS와 IFN-γ에 의해 강화되었다. LPS와 IFN-γ의 조건이 주어지지 않은 상태에서 iNOS mRNA는 젊은 세포에서보다 노화된 세포에서 강하게 발현되었다. 이러한 결과를 통해 세포의 노화가 NO와 iNOS 발현을 증가시킴으로서 치주질환의 진행에 영향을 끼칠 수 있음을 시사하였다.

주요어 : 노화, 사람치은섬유아세포, nitric oxide, inducible nitric oxide synthase