# The Inhibitory Effect of Azithromycin on Cyclosporin A Induced Gingival Overgrowth And its Mechanism of Action

Seong-Hee Park

The Graduate School

Yonsei University

Department of Dental Science

# The Inhibitory Effect of Azithromycin on Cyclosporin A Induced Gingival Overgrowth And its Mechanism of Action

A Dissertation

Submitted to the Department of Dental Science

and the Graduate School of Yonsei University

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy of Dental Science

Seong-Hee Park

December 2004

This certifies that the dissertation of

Seong-Hee Park is approved.

Thesis Supervisor: Kyoo-Sung Cho

Jung-Kiu Chai

Kwang-Kyun Park

Seong-Ho Choi

Won- Yoon Chung

The Graduate School

Yonsei University

December 2004

# 감사의 글

본 논문을 완성함에 있어 시종 간곡하신 지도와 노고를 아끼지 않으신 조규성 교수님께 머리 숙여 감사드립니다. 아울러 많은 조언을 해주신 채중규 교수님, 박광균 교수님, 최성호 교수님, 정원윤 교수님께 진심으로 감사드립니다. 특히 처음부터 끝까지 실험을 맡아 도와주시고 여러가지로 너무나 많이 도와주신 정원윤 교수님께 각별한 감사를 드립니다. 아울러 치주과 교실의 전 의국원들과 더불어 치주과의 제일 어른이신 존경하는 김종관 교수님께 감사의 마음을 전합니다.

끝으로, 항상 사랑으로 곁에서 격려해 주시고 믿어주신 양가 아버님, 어머님, 피곤하고 지친 몸으로 함께 고생해준 사랑하는 내 남편, 듬직한 아들 이준이와 이제 태어난지 5개월 되는 소중한 딸 소정이에게 고맙고 사랑한다는 말을 전하고 싶습니다.

2004년 12월

저자 씀

# Table of Contents

# List of Figures and Tables

**Abstract**

## The Inhibitory Effect of Azithromycin on Cyclosporin A Induced Gingival Overgrowth And its Mechanism of Action

A frequent side effect of cyclosporin A (CsA) administration is gingival overgrowth (GO). Many previous reports have demonstrated that GO can be effectively treated by azithromycin (AZI), a macrolide antibiotic of the azalide subclass. However, the mechanism by which AZI suppresses CsA-induced GO (CIGO) has not yet been elucidated. In the present study, we examined the inhibitory effect of AZI on CIGO and its mechanism of action, and we also investigated the mechanism of CIGO.

Human gingival fibroblasts were isolated from the gingival tissues of healthy subjects and patients exhibiting CIGO. The cell proliferation was significantly increased by CsA exposure for 5 days in fibroblasts isolated from gingival tissue of patients taking CsA. In contrast, AZI significantly inhibited CsA-induced cell proliferation. To identify the differentially expressed genes associated with CsA-induced proliferation in CIGO-GFs, we used differential display-reverse-transcriptase-polymerase chain reaction (DDRT-PCR). In our experimental findings,

seven genes were upregulated by CsA treatment. Among them, the mRNA expression levels of β subunit of the prolyl 4-hydroxylase (P4HB), ribosomal protein L24 (RPL24), ribosomal protein L30 (RPL30) were confirmed by RT-PCR. Upregulated mRNA levels of P4HB, RPL24 and RPL30 by CsA treatment were inhibited dose-dependantly by AZI. The overexpression of P4HB mRNA by CsA shown in our data indicate that CsA may increase the collagen production by stabilizing prolyl 4-hydroxylase, a key enzyme in collagen synthesis and delaying the intracellular degradation of procollagen I. These results suggest that AZI may inhibit CIGO by decreasing CsA-induced cell proliferation and collagen synthesis through downregulation of P4HB in human gingival fibroblasts.

---

**Key words** : cyclosporin A, azithromycin, gingival overgrowth, β subunit of the prolyl 4-hydroxylase (P4HB).

# The Inhibitory Effect of Azithromycin on Cyclosporin A Induced Gingival Overgrowth And its Mechanism of Action

Seong-Hee Park, D.D.S., M.S.D.

Department of Dental Science, Graduate School, Yonsei University

(Directed by Prof. Kyoo-Sung Cho, D.D.S., M.S.D., PhD)

## I. Introduction

Drug-induced gingival overgrowth was first reported in 1939 associated with chronic usage of the anti-epileptic drug phenytoin. Other drugs have now been clearly identified as causing this lesion, notably cyclosporin A and nifedipine (Seymour et al. 1996). Clinically and histologically, gingival overgrowth induced by different drugs, are virtually indistinguishable (Wysocki et al. 1983, Tyldesley & Rotter 1984). In this study, we focused on drug-induced gingival overgrowth by cyclosporin A.

Cyclosporin A (CsA) is a polypeptide composed of 11 amino acids (MW=1202.6) and has been widely used since the 1970s as a immunosuppressive drug to prevent organ transplant rejection and treat various pathological conditions (Schreiber & Crabtree 1992), because of it's low toxicity and potential application in the management of a variety of systemic disorders (Farhad et al. 1990). Despite its

considerable success as a truly selective immunosuppressant drug, CsA treatment may cause various side effects including nephropathy, hypertension, hepatotoxicity, thromboembolic complications, neurotoxicity, hypertricosis and gingival overgrowth (Calne et al. 1979, Atkinson et al. 1984, Vanrenterghem et al. 1985, Tyldesley et al. 1984).

The CsA-induced gingival overgrowth (CIGO) affects the attached gingiva of 25-81% of the treated patients and is observed initially as a papillary enlargement resulting in a lobulated appearance (Bolzani et al. 2000). CIGO appears to be more prevalent in children and adolescents and has a predilection for the anterior gingival tissue. CsA does not promote overgrowth of the oral mucosa of edentulous patients (Seymour et al. 1996). CIGO results in a disturbance in the homeostatic balance which is chracterized by an increase in both the number of fibroblasts and an increase in the volume of the extracellular matrix (ECM). This loss of growth control results in an accumulation of redundant tissue of relatively normal composition (Thomason et al. 1998).

A number of histological and biochemical studies have investigated the changes of cellular function in CIGO. It has been described that CsA affects gingival fibroblast proliferation (Mariotti et al. 1998), the synthesis of collagen and other ECM molecules (Mariani et al. 1996), and also seems to act on ECM degrading enzymes, matrix metalloproteinase (MMP) (Sugano et al. 1998, Arora et al. 2001). The

pathogenesis of CIGO is still uncertain but an appraisal of the available evidence to date would support a multifactorial hypothesis.

Plaque control and removal of local irritants has been shown to be of some benefits in the treatment of CIGO. However, no effective medical treatment is available and gingival surgery is sometimes necessary. Due to the strong propensity for recurrence, the use of intensified conservative periodontal therapy to prevent gingival enlargement in susceptible patients should be encouraged.

Recently Walhstrom et al. (1995) have described improvement of gingival hyperplasia following treatment with azithromycin (AZI), an azalide antimicrobial agent derived from the macrolide antibiotic erythromycin. Although many clinical data have shown the efficacy and safety of AZI (Malizia et al. 1997, Nash et al. 1998, Wirnsberger et al. 1999, Wirnsberger et al. 1998, Citterio et al. 2001, Nowicki et al. 1998, Mesa et al. 2003), the mechanisms of treatment have not been clarified by molecular/ biochemical experiments.

In one of our previous studies, we found that CsA significantly increased cell proliferation and suppressed the mRNA expression and activity of MMP-2 by increasing gene expression of testican1, an extracellular proteoglycan that has been known to inhibit activation of MMP-2 in human gingival fibroblasts (Marr et al. 2000, Mitsutoshi et al. 2003). On the contrary, the combination of AZI and CsA inhibited cell proliferation and the mRNA expression of testican1, influenced the morphology,

recovered the mRNA expression of MMP-2, and increased the activities of MMP-2 and MMP-9. The other study demonstrated that CsA decreased collagen degradation by lowering phagocytic activity of rat gingival fibroblast, and that AZI patially compensated for this lowered phagocytic activity (Paik et al. 2004). These results indicate that AZI inhibits CIGO by suppressing CsA-stimulated cell proliferation and increasing collagen degradation by MMP-2 and phagocytosis.

In our present work, to examine the molecular mechanisms underlying the inhibitory effect of AZI on CIGO, we identified the genes modulating by CsA in human gingival fibroblasts derived from CIGO patients, and investigated the alteration of the identified gene by AZI.

# II. Materials and Methods

## A. Chemicals

Cyclosporin A (CsA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), isopropanol, chloroform, and Tris-Borate-EDTA buffer (TBE) were purchased from Sigma Chemical (St. Louis, MO, U.S.A). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), antibiotic-antimycotic mixture (10,000 units/ml penicillin G sodium, 10,000 $\mu$g/ml streptomycin sulfate, and 25 $\mu$g/ml amphotericin B) and trypsin-EDTA solution (0.25% trypsin, 1mM EDTA) were purchased from Invitrogen Corporation (Grand Island, NY, U.S.A). Fetal bovine serum (FBS) was obtained from the Trace Scientific Ltd. (Melbourne, Australia). Azithromycin dihydrate (AZI) was a generous gift from Pfizer Inc. (Groton, CT, U.S.A).

## B. Cell culture

Human gingival fibroblasts were obtained using primary explant culture of gingival tissues from healthy 5 subjects without evidences of inflammation, hyperplasia, or the history of taking drugs associated with gingival overgrowth, and

from 2 patients with the CsA-induced gingival overgrowth. The donors were from 27 to 65 years old. Informed consent was obtained from each subject. Gingival biopsies were washed with sterile PBS, plated in T-75 flask, incubated in DMEM supplemented with 20% heat-activated FBS and 2% antibiotic-antimycotic mixture at 37°C in a humidified atmosphere with 5% $CO_2$ for 2 weeks. Cells were fed with the fresh medium twice a week. When fibroblasts grew out from the explant, they were trypsinized for secondary cultures. Gingival fibroblasts were seeded in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic mixture in T-75 culture flasks and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C. When cells were grown to confluence, they were detached with 0.25% trypsin-EDTA solution, centrifuged at 1,500 rpm for 3 min, resuspended in DMEM containing 10% DMSO and 30% FBS, and then were stored in liquid nitrogen. All experiments were performed with cells between passage 4 and 6.

**C. Preparation of cyclosporin A and azithromycin**

Due to their highly hydrophobic nature, 10 mg of CsA and 10 mg of AZI were dissolved in 1 ml of DMSO, respectively, and were stored at 4°C until required. The stock solutions were serially diluted in DMEM containing 2% FBS. The final concentrations of CsA or AZI used in the experiments are between 0.1 ng/ml to 100

ng/ml and between 10 ㎍/ml to 100 ㎍/ml, respectively. 0.01% DMSO was used as control.

**D. Treatment of gingival fibroblasts with cyclosporin A and azithromycin**

Human gingival fibroblasts of healthy subjects (GF) or patients with CIGO (CIGO- GF) were seeded at $5\times10^3$ cells/ml with DMEM containing 10% FBS. One day later, the medium was changed with fresh medium supplemented by 2% FBS containing CsA and/or AZI. Gingival fibroblasts were exposed to the various concentrations of CsA (0.1, 1.0, 10, 100 ng/ml), AZI (10, 25, 50, 75, 100 ㎍/ml), or the combination of AZI (10, 25, 50 ㎍/ml) and CsA (10 ng/ml) for 3 days and 5 days.

**E. Cell proliferation**

The MTT colorimetric assay was performed to measure the degree of cell proliferation. Gingival fibroblasts isolated from healthy subjects (GF) or from patients with the CsA-induced gingival overgrowth (CIGO-GF) were seeded at 200 ㎕ ($5\times10^3$ cells/ml) in triplicates in each well of a flat-bottomed 96-well plate and incubated at 37℃ for 24hr. 3-5 days after drug treatment, 50 ㎕ of MTT solution (1 ㎎ dissolved in 200 ㎕ of DMSO) was added to the culture medium in each well

and incubated for 4 h at 37℃ in the plates wrapped with aluminum foil. The medium was removed and MTT-formazan crystals were dissolved by adding 100 $\mu\ell$ of DMSO to each well. Optical density (OD) at 570 nm was measured by ELISA reader (Bio-Rad, U.S.A). Cell proliferation is expressed as the percentage of the OD value related to the control OD value.

**F. Isolation of total RNA**

Total cellular RNA was isolated from CIGO-GFs treated with CsA alone or in combination with AZI. The harvested CIGO-GFs were lysed using TRIzol reagent (Life technologies, Austria), incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complex. After the addition of 0.2 volume of chloroform, samples were shaken vigorously for 15 seconds, incubated for 2–3 minutes, and centrifuged at 12,000 rpm for 15 minutes at 4℃. Total RNA in the upper aqueous phase was precipitated by mixing with an equal volume of isopropanol. The mixtures were then incubated for 10 min at 4℃ and centrifuged at 12,000 rpm for 10 min at 4℃. The pellet was washed with 75% ethanol, dried and dissolved in RNase-free water. To prevent the contamination with chromosomal DNA, total RNA samples were incubated with 10 units of DNase I (GenHunter Corp., Nashville, USA) at 37°C for 30 min, and then DNA-free RNA was isolated using TRIzol reagent. The

concentration and purity of total RNA, and DNase-I treated total RNA were calculated with absorbance at 260 and 280 nm using a spectrophotometer (Pharmacia Biotech, Cambridge, England).

**G. Differential display-reverse-transcriptase-polymerase chain reaction (DDRT-PCR)**

DDRT-PCR was performed using the RNAimage kit (GenHunter Corp.). The DNase I-treated total RNA pools (200 ng per each group) were subjected to reverse transcription in reverse transcriptase buffer (25 mM Tris-HCl, pH 8.3, 37.6 mM KCl, 1.5 mg $MgCl_2$ and 5 mM DTT) with 5 unit /μl of MMLV-reverse transcriptase, 20 μM dNTP mix and 0.2 μM of guanosine-anchored oligo (dT) primer ($HT_{11}$-G). The RT mixture was used for PCR in dilution of 1:10. Subsequent PCR (20 μl) was performed in PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin) containing 2 μM dNTP, 0.2 μM of $HT_{11}$-G, 0.2 μM of arbitrary primer (from H-AP1 to H-AP10), 0.2 μl of α-[$^{33}$P] dATP (2000 Ci/mmole) and 0.05 unit/μl of AmpliTaq DNA Polymerase (Perkin-Elmer). The thermocycler (GeneAmp PCR System 9700, Perkin-Elmer) was programmed as follows: 40 cycles at 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec, and terminated with a final extension at 72°C for 5 min. $^{33}$P-labeled PCR products were separated on 6% denaturing

polyacrylamide gel for 3.5 h at 60 W constant power. The blotted gel on a piece of 3M paper was dried under vacuum at 80°C for 1 h. The autoradiogram oriented with the dried gel was exposed and developed.

**H. Cloning and DNA sequencing**

The interesting cDNA fragments were cut from the dried gel, eluted by boiling in water and reamplified by PCR with the same set of primers at same PCR conditions used in DD-PCR. The reamplified PCR products were cloned in PCR-TRAP vector using PCR-TRAP cloning system (GenHunter) according to the manufacturer's instructions. DNA sequencing for plasmids containing DNA inserts was performed at Takara Korea Biomedical Inc. (Suwon, Korea) and the sequence alignment was performed in GenBank of National Center for Biotechnology Information (NCBI) using standard nucleotide-nucleotide BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLAST/) and all EMBL libraries using Fasta3 program (http://www.ebi.ac.uk/fasta3/).

**I. Primer design and RT-PCR**

Semi-quantitative RT-PCR was performed to confirm the results from DDRT-

PCR. To set the most suitable PCR amplification conditions, primers (Table 1) for interesting gene were determined by an on-line primer design program (Rozen and Skaletsky, 2000). Used primer sets in this study were shown in Table 1. First-strand cDNA was synthesized with 1 μg of total RNAs and 1 μM of oligo-$dT_{15}$ primer using Omniscript Reverse Transcriptase (Qiagen, CA). Using Taq PCR Master Mix kit (Qiagen), subsequent PCR was performed with 0.5 μl of first-strand cDNA and 20 pmole of primers (Table 1). The PCR reaction consisted of initial denaturation at 94°C for 3 min, 3-step cycling (30 cycles) at 94°C for 40 sec, 53°C for 40 sec, and 72°C for 1 min, and final extension at 72°C for 10 min. The amplified PCR products were loaded into 1.2% agarose gel. After ethidium bromide staining, the gel was illuminated on the UV transilluminator and the photography was made using Polaroid DS-34 Instant Camera system (Kodak, USA).

**Table 1. Primers**

| Target genes | | Sequences | Product size(bp) |
|---|---|---|---|
| P4HB | Forward | 5'-GGAGATGACCAAGTACAAGC-3' | 756 |
| | Reverse | 5'-GGCTTTGCGTATTACAGTTC-3' | |
| RPL30 | Forward | 5'-CTCTAGGCTCCAACTCGTTA-3' | 507 |
| | Reverse | 5'-TTAAAGGAAAATTTTGCAGG-3' | |
| RPL24 | Forward | 5'-CAGTTTTAGCGGGTACAAGA-3' | 581 |
| | Reverse | 5'-GAAACTTTCACAGGCTTCAC-3' | |
| GAPDH | Forward | 5'-GTCAGTGGTGGACCTGACCT-3' | 420 |
| | Reverse | 5'-AGGGGTCTACATGGCAACTG-3' | |

**J. Statistical Analysis**

Statistical analysis of the data was performed using the unpaired Student's *t* test. The p value less than 0.05 was considered statistically significant.

# III. Results

## A. The effect of cyclosporin A on the proliferation of GFs and CIGO-GFs

Among 5 GFs isolated from 5 healthy subjects, the proliferation of 3 GFs(GF1, GF2, GF3) were not changed and that of 2 GFs(GF4, GF5) was increased meaningly by CsA treatment for 3 and 5days (Fig.1A). 2 CIGO-GFs from 2 patients with gingival overgrowth (CIGO-GF1, CIGO-GF2) showed significantly increased proliferation when cultured with CsA for 3 and 5 days (Fig. 1B).

## B. The effect of azithromycin on CsA-induced proliferation of CIGO-GFs

To examine the effect of azithromycin on the CsA-induced proliferation of human gingival fibroblast, a highly proliferating CIGO-GF(CIGO-GF1) by CsA treatment was incubated in the 2% FBS-DMEM media in the absence or presence of CsA (10 ng/ml) and azithromycin (10-100 μg/ml) for 5 days. Azithromycin reduced the proliferation of CIGO-GF stimulated by CsA in a dose-related manner as shown in Fig. 2. However, azithromycin alone did not reduced the proliferation of CIGO-GF in 2% FBS media (Fig. 3).

**C. Identification of genes modulating by cyclosporin A or/and azithromycin treatment in CIGO-GFs**

To identify the differentially expressed genes associated with CsA-induced proliferation in CIGO-GFs, DDRT-PCR was performed using 10 sets of primer combination (Fig. 4). As described in Table 2, seven genes were upregulated by CsA treatment. Among them, the mRNA expression levels of β subunit of the prolyl 4-hydroxylase (P4HB) (Fig.5A), ribosomal protein L24 (RPL24) and ribosomal protein L30 (RPL30) (Fig. 6A) were confirmed by RT-PCR.

Furthermore, It was investigated using RT-PCR whether the upregulation of these genes is suppressed by azithromycin. The mRNA levels of P4HB (Fig. 5B), RPL24 and RPL30 (Fig. 6B) upregulated by CsA were inhibited dose-dependently by azithromycin.

**Table 2. Gene expression modified by CsA treatment in CIGO-GFs.**

| Clone No. | Acession No. | Definition | Homology |
|---|---|---|---|
| 3 | NM_000918.2 | Homo sapiens procollagen-proline, oxoglutarate 4-dioxygenase (prolyl 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55) (P4HB), mRNA | 100 |
| 15 | BC030691.1 | Homo sapiens, sparc/osteonectin, cwcv and kazai-like domains proteoglycan (testican), clone MGC:26308 IMAGE:4821322, mRNA, complete cds | 96 |
| 17 | NM_000366.3 | Homo sapiens tropomyosin 1 (alpha) (TPM1), mRNA | 100 |
| 24 | AAN37426.1 | NADH dehydrogenase (ubiquinone) (EC1.6.5.3) chain 2-human mitochondrion, partial (84%) | 94 |
| 33 | M94314.1 | Homo sapiens ribosomal protein L30, mRNA, complete cds | 95 |
| 34 | BC000690.1 | Homo sapiens ribosomal protein L24, mRNA (cDNA clone MGC:2240 IMAGE:3349215), complete cds | 100 |
| 36 | BC040354.1 | Homo sapiens, similar to caldesmon 1, clone MGC:21352 IMAGE:4753285, mRNA, complete cds | 100 |

# IV. Discussion

Gingival overgrowth (GO) has been a recognized side effect of drugs, such as cyclosporin A (CsA), phenytoin, nifedipine, for several years (Rateitachak-Plüss et al. 1983, Kimball 1939, Lederman et al. 1984). Specially, the relationship between CsA treatment and development of gingival overgrowth is well established in humans as well as in experimental models (Bartold 1987, Daley et al. 1984, Nishikawa et al. 1996), although the underlying biochemical mechanisms are not completely understood. This is surprising since CsA is a potent inhibitor of T-cell-mediated immune reactions, and, therefore, presents a unique opportunity to study inflammation in the absence of T-cell activation. Despite extensive studies, over more than half a century, the pathogenesis of CsA-induced gingival overgrowth (CIGO) is uncertain and treatment is still largely limited to the maintenance of an improved level of oral hygiene and surgical removal of the overgrown tissue. Recently, azithromycin (AZI) has been reported to be highly effective in treating CIGO (Malizia et al. 1997, Nash et al. 1998, Wirnsberger et al. 1999, Wirnsberger et al. 1998, Citterio et al. 2001, Nowicki et al. 1998, Mesa et al. 2003). These results imply that AZI has an inhibitory effect on CIGO. However, the mechanism by which AZI suppresses CIGO has not been elucidated yet. Therefore, in the present study, the inhibitory mecanism of AZI on CIGO and its mechanism of action were investigated.

Many previous reports have been described that CsA affects fibroblast proliferation, promotes an abnormal accumulation of extracellular matrix (ECM) molecules in the gingiva and also seems to act on ECM degrading enzymes (Bolzani et al. 2000).

Since the mode of action of CsA is complicated and involves the interaction with the synthesis of several interleukins directly (Powles et al. 1980, Thamson et al. 1983) or indirectly, with monocyte involvement (Reem et al 1983, Hassell et al 1988), it is not clear whether the altered cellular behavior of fibroblast is a direct effect of CsA on fibroblast or whether it is cytokine mediated.

In the present study, we found that the proliferation of 2 CIGO-GFs was significantly increased by CsA tretment in 2% FBS-DMEM. In contrast, cell proliferation in GFs which were isolated from 5 healthy subjects was increased only in 2 GFs. The present data show that the effect of CsA on fibroblast proliferation apparently depends on the individual from whom the strain was derived, the presence of responsive cell subpopulations, and the dose of the drug. That the fibroblasts from different individuals were heterogenous in their proliferative response to CsA may partly explain the observation that not all patients taking CsA develop gingival overgrowth. Such inter-individual susceptibility to these gingival changes may be related to a genetic predisposition.

AZI reduced the proliferation of CIGO-GFs stimulated by CsA in a dose-related

manner. However, AZI itself did not reduced the proliferation of CIGO-GFs in 2% FBS media.

To investigate the molecular events underlying the inhibitory effect of AZI on CIGO, we identified the differentially expressed genes associated with CsA-induced proliferation in a highly proliferating CIGO-GF by CsA, using DDRT-PCR techniques for identifying genes that are over- or under-expressed in one cell or tissue type relative to another (Liang & Pardee, 1992).

As described in our experimental findings, seven genes were upregulated by CsA treatment. The increased mRNA levels of a β subunit of prolyl 4-hydroxylase (P4HB), testican, ribosomal protein L24 (RPL24) and RPL30 were confirmed in the CsA-treated CIGO-GF by RT-PCR. In addition, AZI inhibited the increased mRNA expression of P4HB, RPL24 and RPL30 by CsA.

Ribosomal proteins such as RPL 30 and RPL 24 also have the crucial roles in protein synthesis.

The free β subunit of the prolyl 4-hydroxylase , a tetramer ($\alpha_2\beta_2$), is regarded as the protein disulfide isomerase (PDI) (Myllyharju 2003, Kivirikko & Myllyharju 1998). PDI is a multifunctional protein that both catalyzes the formation of both intra- and interchain disulfide bonds during protein folding and stabilizes prolyl 4-hydroxylase in the cell (Tsai et al. 2001, Wetterau et al. 1991). PDI acts as a molecular chaperone by binding to unfolded proteins, thereby maintaining the

catalytic subunit in a soluble form rather than directly participating in proline hydroxylation for the stabilization of procollagen chains (John et al. 1993). Moreover, a recent study has reported that the association of PDI with procollagen I leads to endoplasmic reticulum retention of procollagen I, which is composed of two proα1(I) chains and one proα2(I) chain and intracellularly degraded immediately after its synthesis, in corneal endothelial cells (Ko et al. 2004). The overexpression of PDI mRNA by CsA shown in our data indicate that CsA may increase the collagen production by stabilizing prolyl 4-hydroxylase, a key enzyme in collagen synthesis and delaying the intracellular degradation of procollagen I. In addition, these results can provide the molecular basis of other in vitro studies that cyclosporin causes a significant increase in the level of type I procollagen (Schincaglia et al. 1992).

Taken together, AZI improves CIGO by inhibiting CsA-induced cell proliferation and collagen production through the downregulation of P4HB.

## V. Conclusion

AZI improves CIGO by inhibiting CsA-induced cell proliferation and collagen production through the downregulation of prolyl 4-hydroxylase β subunit .

## References

Arora PP, Sivestri L, Ganss B, Sodek J, Mcculloch LA. Mechanism of cyclosporin-induced inhibition of intracellular collagen degradation. *J Biol Chem* 2001; 276:14100-14109.

Atkinson K, Biggs J, Darveniza P, Boland J, Concannon A, Dodds A. Cyclosporin-associated central nervous system toxicity after allogeneic bone marrow transplatation. *Transplatation* 1984;38:34-37.

Bartold PM. Cyclosporin and gingival overgrowth. *J Oral Pathol* 1987;16:463-468.

Bolzani G, Colleta RD, Martelli JH, Almeida OP, Graner E. Cyclosporin A inhibits production and activity of matrix metalloproteinases by gingival fibroblasts. *J Periodont Res* 2000;35:51-58.

Calne RY, Rolles K, Thiru S, et al. Cyclosporin A initially as the only immunosuppressant in 34 recipients of cadaveric organ:32 kidneys, 2 pancreas and 2 livers. *Lancet* 1979; 2:1033-1036.

Citterio F, Di Pinto A, Borzi MT, et al. Azithromycin treatment of gingival hyperplasia in kidney transplant recipients is effective and safe. *Transplant Proc* 2001;33:2134-2135.

Daley TD, Wysocki GP. Cyclosporin therapy : It`s significance to the periodontist. *J Periodontol* 1984;55:708-712.

Farhad EB, Terry DR, Anthony MI. Cyclosporin A-induced gingival overgrowth : A comprehensive review. *Quintessence Int* 1990;30:775-783.

Hassell TM, Romberg E, Sobhani S, Lesko L, Douglas R. Lymphocyte-mediated effects of cyclosporin on human fibroblasts. *Transpl Proc* 1988;*XX*(suppl 3):993-1002.

John DC, Grant ME, Bulleid NJ. Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the beta-subunit (PDI) in preventing misfolding and aggregation of the alpha-subunit. *EMBO J* 1993;12 :1587–1595.

Kimball OP. The treatment of epilepsy with sodium diphenyl hydantoinate. *J Am Med Asso* 1939;112:1244-1245.

Kivirikko KI, and Myllyharju J. Prolyl 4-hydroxylase and their protein disulfide isomerase subunit. *Matrix Biol* 1998;16:357-368.

Ko MK, Kay EP. PDI-mediated ER retention and proteasomal degradation of procollagen I in corneal endothelial cells. *Experimental Cell Research* 2004;295:25-35.

Lederman D, Lumerman H, Reuben S, Freedman PD. Gingival hyperplasia associated with nifedipine therapy. Report of a case. *Oral Surg Oral Med Oral Pathol* 1984;57:620-622.

Liang P, Pardee AB. Differintial display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992;257:967-971.

Malizia T, Tejada MR, Ghelardi E, et al. Periodontal tissue disposition of azithromycin. *J Periodontol* 1997;68:1206-1209.

Mariani G, Calastrini C, Carinci F et al. Ultrastructural and histochemical features of the ground substance in cyclosporin A-induced gingival overgrowth. *J Periodontol* 1996;67:21-27.

Mariotti A, Hassell T, Jacobes D, Manning CJ, Hefti AF. Cyclosporin A and hydroxycyclosporin (M-17) affect the secretory phenotype of human gingival fibroblasts. *J Oral Pathol Med* 1998;27:260-266.

Marr HS, Basalamah MA, Bouldin TW, Duncan AW, Edgell CJ. Distribution of testican expression in human brain. *Cell Tissue Res* 2000;302:139-144.

Mesa FL, Osuna A, Aneiros J, et al. Antibiotic treatment of incipient drug-induced gingival overgrowth in adult renal transplant patients. *J Periodont Res* 2003;38:141-146.

Mitsutoshi N, Hisashi M, Junkoh Y, Hiroshi S. Testican 2 abrogates inhibition of membrane-type matrix metalloproteinase by other testican family proteins. *Cancer Research* 2003;63:3364-3369.

Myllyharju J. Prolyl 4-hydroxylase, the key enzymes of collagen biosynthesis. *Matrix Biol* 2003;22:15-24.

Nash MM, Zaltzman JS. Efficacy of azithromycin in the treatment of cyclosporin-induced gingival hyperplasia in renal transplant recipients. *Transplantation* 1998;65:1611-1615.

Nishigawa S, Nagata T, Morisaki I, Oka T, Ishida H. Pathogenesis of drug-induced gingival overgrowth: A review of studies in the rat model. *J Periodontol* 1996;67:463-471.

Nowicki M, Kokot F, Wiecek A. Partial regression of advanced cyclosporin-induced gingival hyperplasia after treatment with azithromycin. A case report. *Ann Transplant* 1998;3:25-27.

Paik JW, Kim CH, Cho KS, Chai JK, Kim CK, Choi SH. Inhibition of cyclosporin A-induced gingival overgrowth by azithromycin through phagocytosis : an *in vivo* and *in vitro* study. *J Periodontol* 2004;75:380-387.

Powles RL, Clink HM, Spence D. Cyclosporin A to prevent graft-versus-host disease in man after allogenic bone marrow transplantation. *Lancet* 1980;*i*:327.

Rateitachak-Plüss E, Heft A, Lörtscher R, Thiel G. Initial observation that yclosporin A induces gingival enlargement in man. *J Clin Periodontol* 1983;10:237.

Reem GH, Cook LA, Vilek J. Gamma interferon synthesis by human thymocytes and T-lymphocytes inhibited by cyclosporin A. *Science* 1983;221:63-65.

Schincaglia GP, Forniti F, Cavallini R, Piva R, Calura G, Del Senno L. Cyclosporin-A increases type I procollagen production and mRNA level in human gingival fibroblasts *in vitro*. *J Oral Pathol Med* 1992;21(4):181-185

Schreiber SL, Crabtree GR. The mechnism of action of cyclosporin A and FK506. *Immunol Today* 1992;13:136-142.

Seymour RA, Thomason JM, Ellis JS. The pathogenesis of drug-induced gingival overgrowth. *J Clin Periodontol* 1996;23:165-175.

Sugano N, Ito K, Murai S. Cyclosporin A inhibits collagenases gene expression via AP-1 and JNK suppression in human gingival fibroblasts. *J Periodont Res* 1998;33:448-452.

Thamson AW, Moon DK, Geczy CL, Nelson DS. Cyclosporin A inhibits lymphokine production but not the responses of macrophages to lymphokines. *Immunology* 1983;48:291-299.

Thomason JM, Sloan P, Seymour RA. Immunolocalization of collagenase(MMP-1) and stromelysin(MMP-3) in the gingival tissue of organ transplant patients medicated with cyclosporin A. *J Clin Periodontol* 1998;25:554-560.

Tsai B, Rodighiero C, Lencer WI, Rapoport TA. Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. *Cell* 2001;104:937–948.

Tyldesley WR, Rotter E. Gingival hyperplasia induced by cyclosporin A. *Br Dent J* 1984;157:305-309.

Vanrenterghem Y, Roels L, Lerut T. Thromboembolic complications and hemostatic changes in cyclosporin-treated cadaveric kidney allograft recipient. *Lancet* 1985;1:999-1002.

Wahlstrom E, Zamora JU, Teichman S. Improvement in cyclosporin-associated gingival hyperplasia with azithromycin therapy. *N Engl J Med* 1995;332:753-754.

Wetterau JR, Combs KA, McLean LR, Spinner SN, Aggerbeck LP. Protein disulphide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. *Biochemistry* 1991;30: 9728–9735.

Wirnsberger GH, Pfragner R, Mauric A, Zach R, Bogiatzis A, Holzer H. Effect of antibiotic treatment with azithromycin on cyclosporin A-induced gingival hyperplasia among renal transplant recipients. *Transplant Proc* 1998;30:2117-2119.

Wirnsberger GH, Pfragner R. Comment on "Efficacy of azithromycin in the treatment of cyclosporin-induced gingival hyperplasia in renal transplant recipients" by Nash and Zaltzman. *Transplantation* 1999;67:1289-1291.

Wysocki GP, Gretzinger HA, Laupacis A, Ular RA, Stillef CR. Fibrous hyperplasia of the gingiva. A side effect of cyclosporin A therapy. *Oral Surg Oral Med Oral Pathol* 1983;55:274-278.

## Figure legends

**Figure 1**. The effect of CsA on the proliferation of GFs and CIGO-GFs.

**Figure 2**. The effect of azithromycin on CsA-induced proliferation of CIGO-GFs.

**Figure 3**. The effect of azithromycin on the proliferation of CIGO-GFs.

**Figure 4.** DDRT-PCR.

**Figure 5**. The gene expression of prolyl 4-hydroxylase β subunit (P4HB).

**Figure 6.** The gene expression of ribosomal protein L24 (RPL24) and RPL30.

Fig. 1. The effect of CsA on the proliferation of GFs and CIGO-GFs

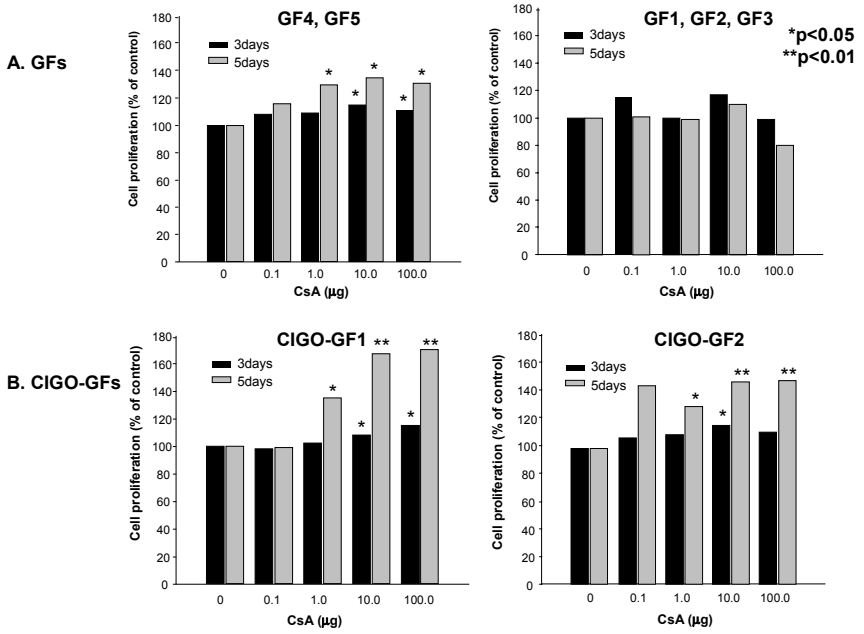
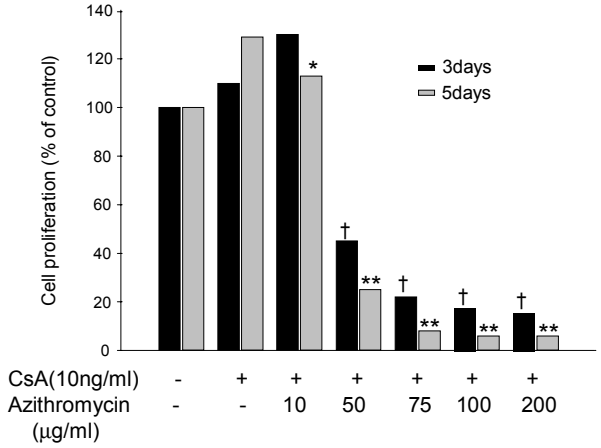



Fig. 2. The effect of azithromycin on CsA-induced proliferation of CIGO-GFs




†p<0.0005; compared to the proliferation of cells treated with CsA alone for 3 days
*p<0.05, p<0.0005; compared to the proliferation of cells treated with CsA alone for 5 days

Fig. 3. The effect of azithromycin on the proliferation of CIGO-GFs

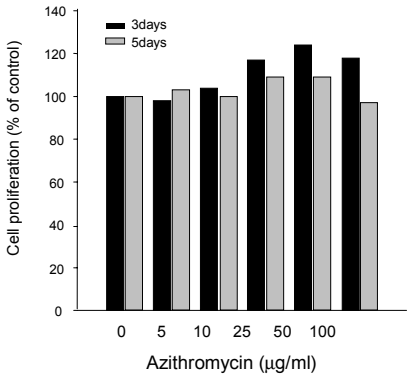



Fig. 4. DDRT-PCR

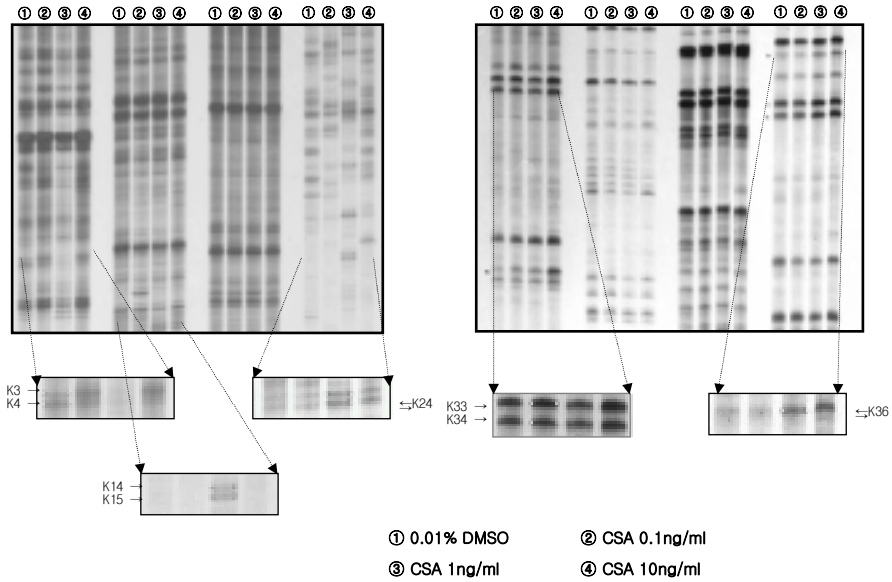


Fig. 5. The gene expression of prolyl 4-hydroxylase β subunit (P4HB)

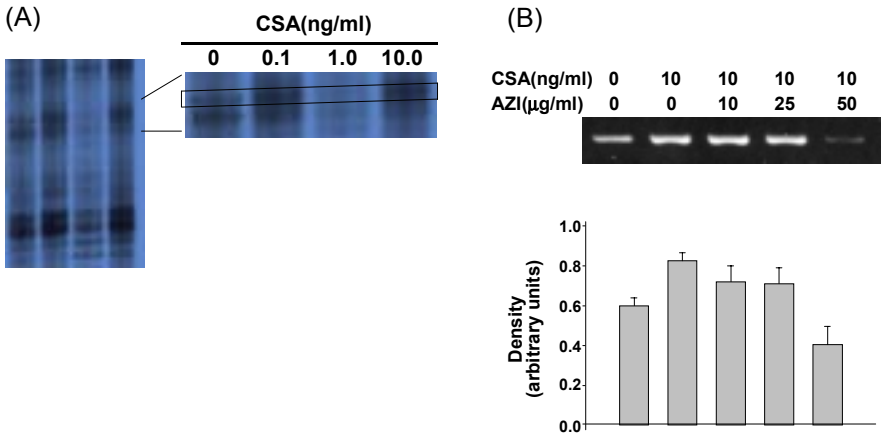
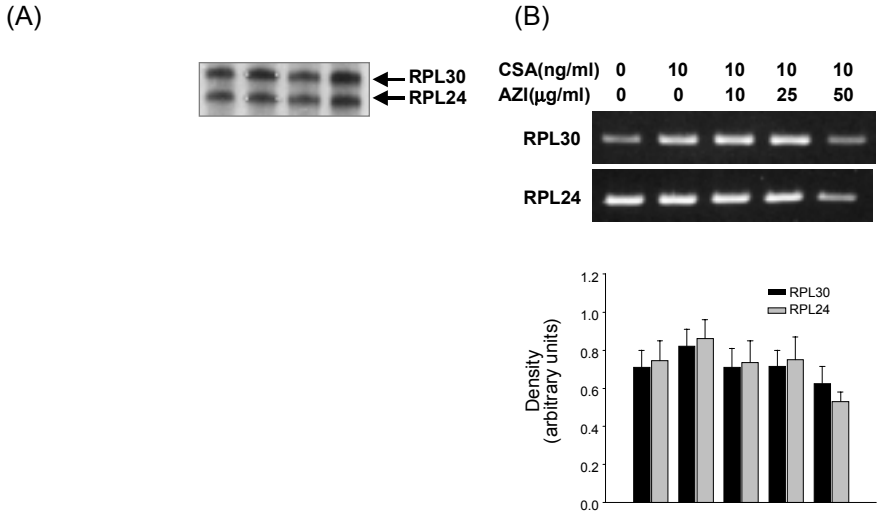



Fig. 6. The gene expression of ribosomal protein L24 (RPL24) and RPL30



국문요약

# Cyclosporin A에 의해 유도된 치은 과성장에 대한 azithromycin의 억제 효과 및 작용기전에 대한 연구

<지도 교수 : **조 규 성**>

연세대학교 대학원 치의학과

**박 성 희**

**목적** : 치은 과성장을 일으키는 대표적인 약물로는 면역억제제로 많이 사용되고 있는 cyclosporin A (CsA), 항경련제로 쓰이는 phenytoin, 고혈압치료제로 사용되는 nifedipine을 들 수 있다. 보통 이러한 치은 과성장은 통상적으로 치은 절제술을 통해 치료하고 있지만 잦은 재발로 인해 정기적인 수술을 필요로 하여 환자들이나 의사들에게 큰 부담을 주고 있다. 최근 임상연구를 통해서 CsA 에 의한 치은 과성장이 macrolide 계열의 항생제인 azithromycin (AZI)에 의해 효과적으로 억제된다는 결과가 보고되었다. 그러나 현재까지 CsA에 의한 치은 과성장의 유도기전과 AZI에 의한 억제기전은 명확히 밝혀지지 않고 있다. 이에 본 연구에서는 CsA에 의한 치은 과성장의 유도기전과 AZI에 의한 억제기전을

분자 생물학적 관점에서 밝혀보고자 한다.

**방법** : 치은 섬유아세포는 과거에 CsA를 복용하여 치은과성장을 보인 환자와 CsA를 복용한 경험이 없는 건강한 환자의 치은에서 explant culture로 분리하였다. CsA에 대한 치은 섬유아세포의 증식률과 AZI에 의한 억제율은 MTT assay에 의해 측정하였다. CsA에 의한 세포증식률이 가장 높은 치은섬유아세포에 CsA를 처리한 후 differential display-reverse transcriptase-polymerase chain reaction(DDRT-PCR) 방법을 수행하고 DNA sequence를 조사하여 유전자 발현의 변화를 조사하였다. 의미있다고 여겨지는 세가지 유전자, prolyl 4-hydroxylase의 β subunit (P4HB), ribosomal protein L24 (RPL24), ribosomal protein L30 (RPL30)의 발현을 RT-PCR로 확인하였으며, CsA에 의해 증가된 이들 유전자의 발현에 대한 AZI의 영향을 RT-PCR로 조사하였다.

**결과** : CsA에 의한 세포의 증식률은 건강한 환자보다 CsA를 복용한 경험이 있는 환자로부터 분리한 치은 섬유아세포에서 뚜렷하게 증가되었다. 그러므로 이후의 연구는 CsA를 복용한 경험이 있는 환자의 치은 섬유아세포로 진행하였다. 5일간 CsA(10ng/ml)와 AZI을 함께 처리한 경우, AZI의 농도에 비례하여 치은 섬유아세포의 증식률이 감소하였다. CsA를 처리한 치은 섬유아세포에서의 DDRT-PCR 결과 collagen 생합성과 세포의

증식에 영향을 미치는 P4HB, RPL24, RPL30 유전자의 발현양이 CsA에 의해 현저하게 증가됨을 볼 수 있었다. 그러나, CsA와 함께 AZI을 처리했을 경우에는 CsA에 의해 증가된 이러한 유전자의 발현양이 AZI에 의해 억제되는 것으로 관찰되었다.

**결론** : AZI이 CsA에 의해 증가된 세포증식과 P4HB 유전자의 발현양을 감소시켜 collagen의 합성을 감소시킴으로써 CsA에 의해 유도된 치은과성장을 억제하는 것으로 사료된다.

---

핵심되는 말 : cyclosporin A, azithromycin, 치은과성장, prolyl 4-hydroxylase의 β subunit