The Effect of Azithromycin on The Cyclosporin-A Induced Gingival Fibroblast Overgrowth *in vitro*

연세대학교 대학원

치의학과

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이 논문을 석사 학위논문으로 제출함

2000년 12월 일

연세대학교 대학원

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노현수의 석사 학위논문을 인준함

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본 논문이 완성되기까지 너무나 많은 교수님들과 동료, 선·후배 여러분께서 도와주셨습니다. 바쁘신 와중에도 꼼꼼히 가르쳐주신 박광균 교수님께 깊은 감사를 드립니다.

김종관 교수님께서는 연구자가 가져야 할 끊임없는 창조적 자세를, 이승일 교수님께서는 연구 결과에 대한 냉철한 비판적 자세를, 채중규 교수님께는 밀도있는 검증의 자세를, 조규성 교수님께서는 일을 진행해 나가는데 있어서 중심의 역할을, 최성호 교수님께서는 기획력과 추진력을, 유윤정 교수님께서는 학문적 이해의 중요성을, 서정택 교수님께서는 실험을 구체화하는 방법을, 최봉규 교수님께서는 실질적인 실험 방법을 가르쳐 주셨습니다.

처음 실험을 함에 있어서 많은 도움을 주신 박미영, 문석준, 김윤주 선생님과 교정 작업을 도와주신 백정원 선생님께도 감사의 말씀을 드립니다. 지면상 열거하지 못했지만 그 외의 많은 치주과, 구강생물학교실 교실원 여러분들께 감사드립니다.

언제나 큰 사랑과 염려를 베풀어주신 양가 부모님과, 누님, 매형, 그리고 사랑하는 아내 수진에게 이 논문을 드립니다.

이 논문을 시작으로 더욱 학문에 매진하는 모습을 보여드리도록 노력하겠습니다. 모든 분들께 진심으로 감사드립니다.

2000년 12월

저자씀

목 차

도 표 목 차

국문요약

Azithromycin이 Cyclosporin-A에 의한 치은 섬유아세포 과증식에 미치는 영향에 대한 *in vitro* 연구

Cyclosporin-A(CsA)는 장기와 조직 이식에 따른 거부반응을 조절하기 위해 사용되는 면역억제제로, 이식의학의 발달과 더불어 사용량이 증가하고 있다. CsA의 부작용중의 하나인 치은과증식은 30-50%의 빈도로 발발하고 있다. 최근 macrolide 계열의 항생제인 azithromycin을 이용하여 이런 부작용을 억제시킨다는 임상 보고가 있어서, 이를 실험적으로 확인하고저 하였다.

이를 위해 CsA를 투여한 적이 없는 환자에서 정상 치은조직을 채취, 치은 섬유아세포를 배양하였다. 우선 CsA에 대한 치은 섬유아세포의 반응을 보기 위해 다양한 농도(10^{-8}-10^{-10}g/㎖)로 처치하여, 세포 증식량과 교원질 합성량을 MTT assay와 Sircol Collagen Assay®를 이용하여 측정하였다. 이에 반응을 보인 조건과 세포를 대상으로 다양한 농도(10^{-5}-10^{-8}g/㎖)의 azithromycin을 CsA와 동시 처치하여 아래와 같은 결과를 얻었다.

1. CsA는 일부 치은 섬유아세포의 증식을 증가시켰다. 그러나 Collagen 합성능에는 변화를 주지 않았다.

2. Azithromycin은 정상 치은 섬유아세포의 증식능에 영향을 주지 않았다.

3. Azithromycin은 CsA에 반응을 보인 세포의 증식을 감소시켰으며, 이는 정상 수준과 유사하였다.

이상의 결과에서 azithromycin이 CsA에 의한 치은과증식 치료에 유익하다고 사료된다.

핵심되는 말 : Azithromycin, Cyclosporin-A, 치은섬유아세포, 치은과증식, 증식률, Collagen.

The Effect of Azithromycin on The Cyclosporin-A Induced Gingival Fibroblast Overgrowth *in vitro*

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I. Introduction

Cyclosporin-A(CsA) is an immuno-suppressant drug widely used for the control of rejection phenomena following organ and bone marrow transplantation.[7,8] The success achieved with CsA in transplant medicine and the wide variety of systemic disorders of immunologic origin treated by or potentially treatable by CsA lead to estimate that one billion persons worldwide will be taking CsA within the next decade.[7,8] One of the most prominent side effects of CsA therapy is gingival overgrowth or hyperplasia[17,20,21,23] with a generally accepted incidence of approximately 30 to 50%.[8,16,17,23] The cellular/molecular mechanism of action relative to connective tissue hyperplasia are poorly understood.[9]

There have been some clinical studies which suggested that a gingival overgrowth can be effectively treated by the azithromycin, a macrolide antibiotics of the azalide subclass with a long half-life while CsA was continued.[14,24,25]

Wirnsberger et al. reported that the gingival overgrowth induced by the immuno-suppressant, CsA could be effectively treated with a single course of azithromycin in almost all cases.[25]

Nash et al. studied about the efficacy of azithromycin to treat cyclosporin-induced gingival hyperplasia in renal transplant recipients, and reported that the reduction of gingival tissue was observed.[14] The similar results are found in the other papers by Boran et al.,[4] Puig et al.,[19] Gomez et al.,[6] Jucgla et al.,[10] Palomar et al.,[18] and Nowicki et al.[15] There are many clinical papers about the efficacy of azithromycin. But the results are not confirmed by experiments.

The present study was undertaken to assess the effect of azithromycin on the CsA induced gingival fibroblast overgrowth *in vitro*.

II. Materials and Methods

Minimum essential medium(MEM) with Earle's salts, L-glutamine, and non-essential amino acids(Lot No. 1018860), antibiotic-antimycotic prepared with penicillin G sodium, streptomycin sulfate and amphotericin B(Lot No. 1020066) and Trypsin-EDTA(Lot No. 1027232) were all purchased from Life Technologies(Grand Island, N. Y. 14072 USA). Fetal bovine serum(FBS, Lot No. 907040-500) was purchased from the Trace Scientific Ltd., Melbourne, Australia. The Sircol Collagen Assay® kit was purchased from the Biocolor Ltd., Belfast, N. Ireland. Cyclosporin-A(Lot No. 49H4066) and MTT solution (Thiazolyl blue, Lot No. 66H50336) were purchased from Sigma Chemical, St. Louis MO USA. Azithromycin(Azithromycin dihydrate, Lot No. 25381-087-08) was a generous gift from Pfizer Inc., Groton CT USA.

Culture of Fibroblasts

Human gingival fibroblasts were isolated from explant cultures of healthy gingiva from normal patients who had no history of taking CsA before. The cells were maintained in the minimum essential medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic mixture (10 000 units/㎖ penicillin G sodium, 10 000 ㎍/㎖ streptomycin sulfate, 25 ㎍/㎖ amphotericin). Fibroblasts of passage No. 3-5 were used in every experiments.

Preparation of Azithromycin

Azithromycin was soluble in culture media. An 1㎎/㎖ solution was made by dissolving 1㎎ azithromycin in 1㎖ culture media, then diluted in the media to reach the final concentrations. The final concentrations used in the experiments are between 10^{-5} to 10^{-8} g/㎖.

Preparation of Cyclosporin-A

Due to its highly hydrophobic nature, CsA is insoluble in culture media. Therefore, before mixing CsA into culture system, an 1㎎/㎖ solution was made by dissolving 1㎎ CsA in 100㎕ ethanol, then 10㎕ Tween 20 was added while vigorously shaking. Finally, 890㎕ of the culture media to be used was added. The sample was then diluted in the media to reach the final concentrations. The final concentrations used in the experiments are between 10^{-8} to 10^{-10}g/㎖. Appropriate solvent controls were run and no detectable effect on the cells was noted(results not shown).

Fibroblasts Proliferation Assay

To measure the effect of CsA and azithromycin on gingival fibroblasts proliferation, human gingival fibroblasts of passage No. 3-5 were seeded, in triplicate, into 96-well plates at an initial density of 10 000 cells per well and allowed to attach and spread for 1 day in MEM containing 10% FBS. The medium was then replaced with a range of concentrations of CsA and azithromycin in 100㎕ MEM containing 2% FBS. After 48 hours incubation at

37℃ in a moist atmosphere of 5% CO_2 and 95% air, the medium was replaced with 80㎕ MEM containing of 2% FBS and 20㎕ MTT solution(5㎎/㎖). The cells were incubated for a further 4 hours. The medium was removed and 100 ㎕ DMSO was added. Samples were assessed for MTT solution reaction under 530nm photospectrum. The results(optical density) were believed to reflect the cell proliferation capacity.

Analysis of Collagen Synthesis

Collagen synthesis by gingival fibroblasts was assessed using the Sircol Collagen Assay® kits. Briefly, triplicate cultures of confluent cells in 96-well plates were incubated in the presence of 2% FBS alone or 2% FBS and various concentrations of CsA. 1.5㎖ capacity conical microcentrifuge tubes are added 100㎕ test samples from each well and 100㎕ Sircol dye reagent. The tubes were mixed gently at the room temperature for 30 minutes and then centrifuged at 5000 x g for 10 minutes. The supernatant was drained off and 100㎕ of the alkali reagent was added. Samples were assessed under the wave length of 530nm using ELISA reader(DYNATECH lab., VA USA). Using the straight line calibration curve, collagen synthesis amount was measured.

Light Microscopy

Gingival fibroblasts were cultured in 2 different TC-flask, and when the cells filled about 70% of the flask, the media of one flask was replaced with 10㎖ MEM containing 2% FBS, the other with 10㎖ MEM containing 2% FBS and CsA at the concentration of 10^{-9}g/㎖. The cells were incubated for a further 2 days. The flasks were examined with a light microscope to examine if there is a morphological change.

Statistical analysis

All data were subjected to statistical analysis using the method of Kruskal-Wallis test.

III. Results

1. Fibroblasts proliferation to CsA

Since CsA-associated gingival overgrowth could be due to a proportional increase in both cell numbers and tissue mass, the proliferation and the protein synthetic capacity(especially the collagen synthesis) according to the various concentrations of CsA were assessed by MTT assay and commercial collagen assay kits, respectively.

CsA appeared to stimulate the proliferation of primary cultured human gingival fibroblasts. Especially at the concentration of 10^{-9}g/㎖, maximum stimulation was evident(Fig. 1). Furthermore this stimulation occured within the concentration range found in plasma and tissues of patients taking CsA and thus highlights the likelyhood of biological activity of CsA toward gingival fibroblasts *in vivo*.[1)]

But such a tendency did not occured in every primary cultured human gingival fibroblasts. Gingival fibroblasts were gained from 9 normal patients, and 4 of them showed the stimulation to CsA, the others vice versa(Fig. 1).

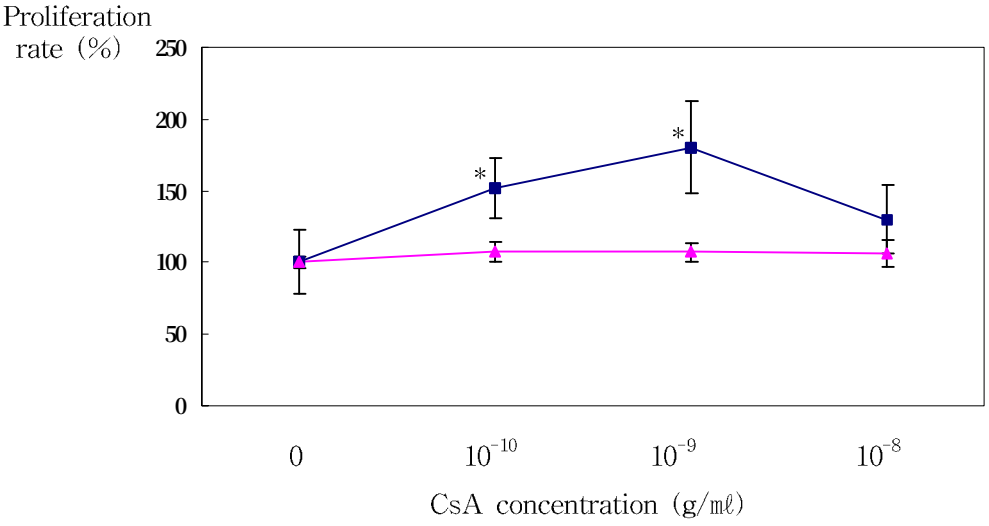


Fig. 1. The effect of CsA on the proliferation of human gingival fibroblasts

■ : cells stimulated by CsA

△ : cells less stimulated by CsA

(Statistically significant difference($p<0.05$) is indicated by '*')

The gingival fibroblasts proliferation was assessed using MTT assay, and the result (O. D. value) was converted to the proliferation percent (the control was assigned as 100%).

2. Collagen synthesis to CsA

In addition to an assessment of cellular proliferative activity, the effect of CsA on the biosynthesis of collagen was also measured. However the activity according to various concentrations of CsA was not statistically significantly different(Fig. 2). Both gingival fibroblasts stimulated and less stimulated by CsA had the same tendency in common.

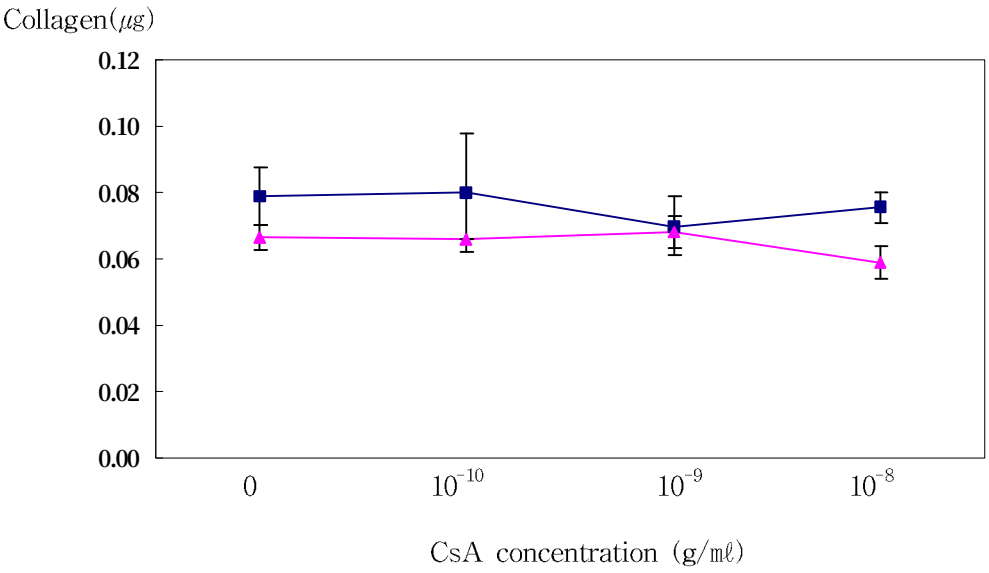


Fig. 2. The effect of CsA on the collagen synthetic ability of human gingival fibroblasts.

■ : cells stimulated by CsA

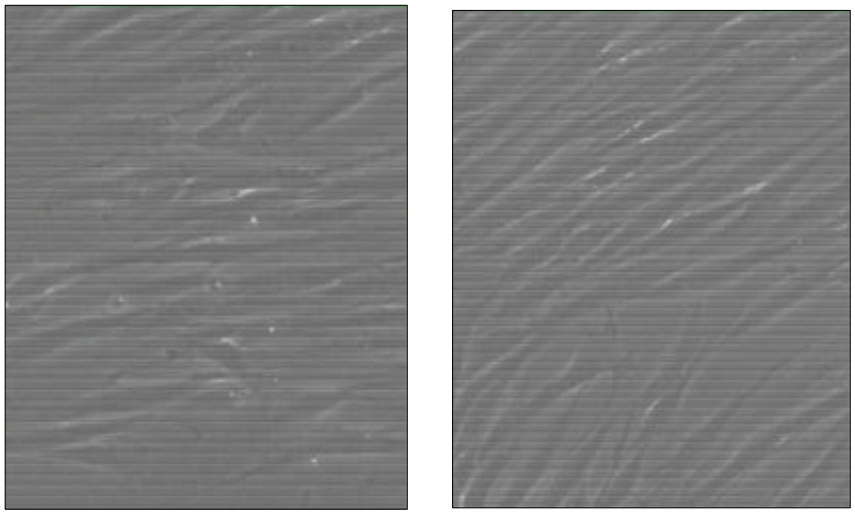
△ : cells less stimulated by CsA

(There was no statistically significant difference($p<0.05$))

Collagen synthesis was assessed by Sircol Collagen Assay® kit.

3. Light microscopic view to CsA

There was no evidence of morphological change when the gingival fibroblasts were cultured with CsA. Compared with gingival fibroblasts without CsA, gingival fibroblasts with CsA showed more proliferation(Fig. 3).



A B

Fig. 3. The gingival fibroblasts cultured with or without CsA (x400)
A : without CsA,
B : with CsA at the concentration of 10^{-9}g/㎖

4. The effect of azithromycin on the proliferation of gingival fibroblasts

Primary cultured gingival fibroblasts stimulated by CsA were selected and tested for assessing the effect of azithromycin.

At first in order to determine if azithromycin originally has an effect on the normal conditioned gingival fibroblasts, various concentrations of azithromycin were treated (Fig. 4). The slight elevation of the gingival fibroblasts proliferation was shown at the concentration of 10^{-7}, 10^{-8}g/㎖, but it was not statistically significant compared with the control.

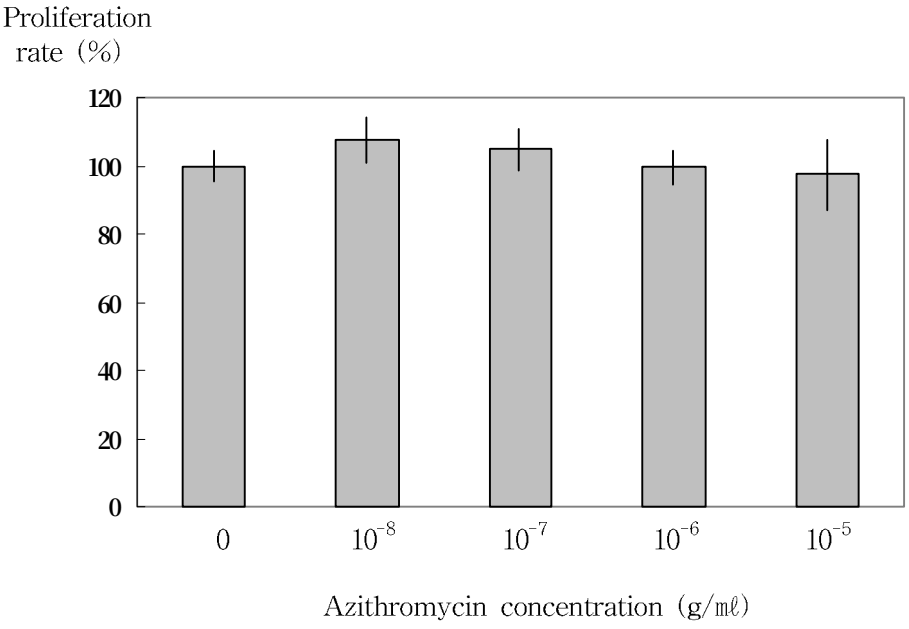


Fig. 4. The effect of azithromycin on the proliferation of human gingival fibroblasts which are treated without CsA

5. Inhibitory effect of azithromycin on CsA induced fibroblast proliferation

To determine whether azithromycin has an inhibitory effect on CsA induced gingival fibroblasts overgrowth, various concentrations of azithromycin with CsA at the concentration of 10^{-9}g/㎖ were treated (Fig. 5).

Gingival fibroblasts proliferation was decreased compared with CsA treated control, and especially at the concentration of 10^{-6}, 10^{-8}g/㎖ azithromycin, statistically significant decrease was noted. But the decreased level was similar with the control without CsA and azithromycin.

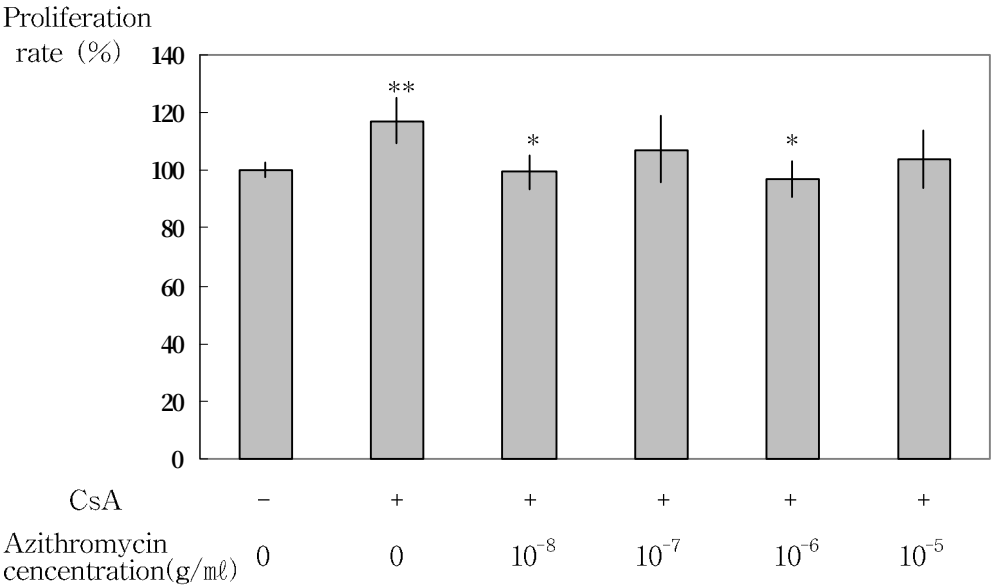


Fig. 5. The effect of azithromycin on the proliferation of human gingival fibroblasts which are treated with CsA at the concentration of 10^{-9}g/㎖

* means the statistically significant decrease($p<0.05$) compared with the control with CsA

** means the statistically significant increase($p<0.05$) compared with the control without CsA

IV. Discussion

The present study analyzed the effect of azithromycin on the CsA treated gingival fibroblasts. Azithromycin has been reported to have an inhibitory effect on CsA induced gingival hyperplasia since 1985.[24] Some clinical studies have supported these findings.[14,25] But the reason gingival hyperplasia happens to the patients taking CsA is not yet clear, the mechanism of azithromycin is not yet known. We tried to find out the possible mechanisms.

For this purpose, gingival fibroblasts have been examined with respect to their proliferative and synthetic activities at first. Because hyperplasia is defined as an increase in the size of an organ or tissue due to an increase in the number of its specialized constituent cells and interstitial cellular matrix mainly composed of collagen about 60%.[5,12]

Nine primarily cultured cell lines were assessed for the reaction to CsA, some of them showed the stimulated proliferation, but the others did not. This is perhaps due to the phenotypic difference among fibroblast cell lines, considering the fact that the gingival hyperplasia as a side effects of CsA has about 30 to 50% incidence for the patients.[8,16,17,23]

According to the results of the experiments, in the fibroblasts which responded to CsA, CsA seems to have the capacity to stimulate fibroblasts proliferation. But it does not have the tendency to stimulate the collagen synthesis activity. The results comply with previous research results.[2,3]

There are so many suggestions why gingival overgrowth occurs to patients taking CsA, these observations imply that stimulation of fibroblasts proliferation could be a major contributory factor in the pathogenesis of CsA induced gingival overgrowth.

In order to assess the inhibitory effects of azithromycin on CsA induced gingival hyperplasia, gingival fibroblast cell lines which were stimulated by CsA were treated with azithromycin and the proliferation was measured. The concentration of azithromycin was decided due to the concentration in periodontal tissues.[11)]

When azithromycin was treated with CsA at the concentration which exhibits the maximum stimulation of fibroblasts proliferation, inhibitory effect of azithromycin was shown. The fact that fibroblast proliferation displayed significant decrease supports the clinical reports that azithromycin has an inhibitory effect on CsA induced gingival hyperplasia.[4,6,10,14,15,18,19,25)]

It is difficult to explain the mechanism underlying this desirable effect. One possible reason may be due to the stimulated fibroblasts proliferation, and this is controlled by a multitude of cytokines(eg, interleukins, TGF-β) produced by inflammatory cells, epithelial and endothelial cells, and fibroblasts themselves.[13,22)] Azithromycin is one of the antibiotics and the concentration of this antibiotic in periodontal tissues is high.[11)] It is easy to infer that azithromycin reduces the number of bacteria and inflammation, and that inflammatory cytokines would be reduced. It is easy to say that "That's why azithromycin is effective in CsA-induced gingival hyperplasia"

But the clinical situation like inflammation is excluded in this study design, so a kind of direct interaction of azithromycin with fibroblasts and / or CsA seems to cause such a inhibitory effect, which is not just because of reduction of inflammation.

Further study will focus on the effects of azithromycin on cytokines, growth hormones, and products from fibroblasts.

V. Conclusion

The study was undertaken to assess the effect of azithromycin on the proliferation of the gingival fibroblasts which are stimulated by CsA. First the proliferation and the collagen synthetic capacity of gingival firboblasts were assessed. After that, azithromycin was treated in the gingival fibroblast cell lines which responded to CsA and the proliferation was measured. The results are as follows.

1. CsA stimulated the proliferation of some of the gingival fibroblasts, but did not alter the collagen synthetic ability

2. Azithromycin did not alter the normal proliferation of the gingival fibroblasts.

3. Azithromycin has the inhibitory effects on the proliferation of the gingival fibroblasts which are stimulated by CsA.

On the basis of these results, the azithromycin therapy is beneficial on the CsA induced gingival hyperplasia.

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ABSTRACT

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Cyclosporin-A is an immuno-suppressant drug widely used for the control of rejection phenomena following organ and bone marrow transplantation. The success achieved with CsA in transplant medicine lead so many patients to take CsA. One of the most prominent side effects of CsA therapy is gingival overgrowth or hyperplasia with a generally accepted incidence of approximately 30 to 50%. Recently there have been some clinical papers which suggested that a gingival overgrowth can be effectively treated by the azithromycin, macrolide antibiotics. The present study was undertaken to assess the effect of azithromycin on the CsA induced gingival fibroblasts overgrowth *in vitro*.

For this purpose, the human gingival fibroblasts were isolated from explant cultures of healthy gingiva from 9 patients who had no history of taking CsA before. At first, various concentrations(10^{-8}-10^{-10}g/㎖) of CsA were treated to assess the proliferation and collagen synthesis using MTT assay and Sircol

Collagen Assay® kit respectively. Various cencentrations (10^{-5}-10^{-8}g/㎖) of azithromycin were co-treated with CsA to the cells responded to CsA, and proliferation was assessed.

The results are as follows.

1. CsA stimulated the proliferation of some of the gingival fibroblasts, but did not alter the collagen synthetic ability

2. Azithromycin did not alter the normal proliferation of the gingival fibroblasts.

3. Azithromycin has the inhibitory effects on the proliferation of the gingival fibroblasts which are stimulated by CsA.

On the basis of these results, the azithromycin therapy is beneficial on the CsA induced gingival hyperplasia.

Key words : Azithromycin, Cyclosporin-A, gingival fibroblasts, gingival overgrowth, proliferation rate, Collagen.