

A Novel Method to Study the Effects of Cyclosporine on Gingival Overgrowth in Children

Keumah Han¹, Jongsoo Kim²

¹*Department of Periodontology, Sejong Dental Hospital, School of Dentistry, Dankook University*

²*Department of Pediatric Dentistry, School of Dentistry, Dankook University*

Abstract

Previous studies to elucidate the etiology of cyclosporine(Cs)-induced gingival overgrowth in children have not completely excluded all factors that may cause differences among individuals. This study examined the effect of cyclosporine on the metabolism of type 1 collagen(CoL-I) in experimental models that controlled the effects of biological variations on individuals. Five 5-week-old male Sprague-Dawley rats were administered Cs by gastric feeding for 6 weeks. Gingival specimens were harvested from the mandibular posterior area before beginning Cs administration and at 2, 4, and 6 weeks thereafter. Gingival fibroblasts were cultured from all the 20 biopsies collected from the gingiva. Half of the fibroblasts collected prior to the Cs administration were designated as Control. The other half of the fibroblasts were treated with Cs in vitro and called in vitro test group(Tt). The fibroblasts collected 2, 4, and 6 weeks after the Cs administration were called in vivo test groups : T2, T4, T6, respectively. Immunofluorescence microscopy was used to detect CoL-I in all the fibroblasts. CoL-I was analyzed at both the gene and protein expression levels by real-time polymerase chain reaction and western blotting. Changes in CoL-I before and after Cs treatment were evaluated from the gingiva of each rat. There was no significant difference in gene expression of CoL-I in the control and test groups. CoL-I protein expression levels of fibroblasts increased in in vitro Cs treatment for each individual, and also increased in in vivo Cs treatment. In this study, the experimental method that control biological variations that can occur due to differences among individuals was useful. Subsequent studies on other factors besides CoL-I and in-depth studies in humans are needed.

Key words : Biological variation, Collagen type I, Cyclosporine, Fibroblast, Gingival overgrowth

I. Introduction

Cyclosporine (Cs) is an immunosuppressant used for preventing graft rejection and treating autoimmune diseases[1]. Gingival overgrowth (GO) is an adverse effect associated with Cs administration. It is frequent in adolescents and children as

well as in adults. The prevalence of GO in young patients is 13 - 85%[2]. GO is a serious intraoral complication that is difficult to treat completely without discontinuing the drug; therefore, it presents difficulties in maintaining oral health and has a negative impact on the overall health of the patient. Since GO does not occur in all patients, identification of patients with

Corresponding author : Jongsoo Kim

Department of Pediatric Dentistry, School of Dentistry, Dankook University, 119, Dandae-ro, Dongnam-gu, Cheonan, 31116, Republic of Korea

Tel: +82-41-550-0223 / Fax: +82-41-551-1935 / E-mail: jskim@dku.edu

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an increased risk of GO is important when prescribing immunosuppressants. This should be a consideration especially for oral health of young patients. It's because it can have a greater impact on children and adolescents who suffer from such adverse effects from an early age. To date, several studies and clinical approaches have attempted to clarify the pathogenesis of Cs-induced GO, and extracellular matrix accumulation has been suggested as the most important event. Studies on type I collagen (CoL-I), which is the main extracellular matrix component, have focused on CoL-I metabolism and matrix metalloproteinase, which are the main targets of Cs[3,4]. A limitation of these studies is that different individuals were assigned to the control group and test group for investigating the effect of Cs on CoL-I metabolism. Because these studies did not control the biological variation between individuals, the effects of factors other than CoL-I cannot be dismissed. Therefore, the study design needs to be modified to identify the effect of Cs on CoL-I metabolism accurately, with all the other factors controlled. The present study addressed the limitations of previous studies by using a novel approach, wherein the experiment was conducted on the same individuals to control variability between individuals

II. Material and methods

All of the animals and protocols used in this study were approved by the Institutional Animal Center and Use Committee, Ajou University Medical Center (approval No.: IA-CUC-2013-0031).

1. Animals and gingival specimens

Five 5-week-old male Sprague-Dawley rats weighing 150 - 160 g were used (designated as A1 - A5). The animals received Cs (10 mg/kg body weight) in corn oil daily by gastric feeding for 6 weeks[5]. The Cs was solution (Sandimmune Neoral® Solution, Novartis Korea, Seoul, South Korea) (10 g/100 mL). The rats were anesthetized by intraperitoneal injection of a combination of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50®, Virbac Korea, Seoul, South Korea) (25 mg/kg) and Xylazine (Rompun®, Bayer, Leverkusen, Germany) (10 mg/kg), and samples of their gingival tissues (3.0 mm × 2.0 mm × 0.5 mm, width×height×depth) were taken from the buccal area of mandibular first and second molars. Specimens harvested before the commencement of Cs treatment served as Controls.

All gingival specimens were obtained 2, 4, and 6 weeks after the commencement of Cs treatment; these were designated as *in vivo* test groups Tv2, Tv4, and Tv6, respectively. All of the animals were sacrificed at week 6.

2. Preparation of gingival fibroblasts

Rat gingival fibroblasts were prepared according to a modified version of a previously described method[6]. The gingival specimens were placed in Hank's balanced salt solution (GIBCO-BRL, Grand Island, NE, USA) containing penicillin/streptomycin. They were minced into smaller pieces and digested in a solution of 4 mg/mL type 1 collagenase, and 3 mg/mL dispase for 3 hours at 37°C. Single-cell suspensions were obtained by passing the solution through a 70-µm strainer. Monolayer cultures were prepared using conventional techniques in Dulbecco's modified Eagle's medium (WELGENE, Daegu, South Korea), supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The culture medium was refreshed every 2 - 3 days. Cells at passages 3 and 4 were used for further *in vitro* study.

3. *In vitro* Cs treatment of cultured cells

Some of the cells from Controls were separated for *in vitro* Cs treatment and assigned to the *in vitro* test group (designated as Tt). A stock solution of 1 mg/mL was prepared by dissolving 1 mg of Cs in 45 µL of ethanol and 5 µL of Tween-20 as cosolvents[7]. In consideration of Cs being used clinically at concentrations ranging from 250 to 400 ng/mL, and at a maximum dose of 1000 ng for *in vitro* studies[8], a mid-range concentration of 700 ng/mL was chosen for the present study. The medium was then added to give a final volume of 1.0 mL. Subconfluent cultures were then maintained (24 hours) in serum-free medium before addition of Cs (700 ng/mL). When the gingival fibroblasts were close to confluence (i.e., at 80%), Cs was added to the cultures, which were then incubated at 37°C for 6 hours[8]. The cell cultures were trypsinized and the fibroblasts were collected by centrifugation at 1610 × g for 5 min. They were finally washed in phosphate-buffered saline (PBS) and harvested by further centrifugation (also 1610 × g for 5 min).

4. Immunofluorescence analysis

Immunofluorescence staining was conducted to enable observation of CoL-I. After culturing the Control, Tv2, Tv4 and Tv6 samples including Tt, the cellular samples were fixed with formaldehyde and dehydrated with ethanol. They were then washed with Dulbecco's phosphate-buffered saline (DPBS) (WELGENE) and incubated with the primary antibody (Polyclonal goat CoL-I, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 1 : 200 at 4°C. The cells were washed with DPBS and incubated in fluorescein isothiocyanate (FITC)-labeled mouse antigoat IgG and FITC-labeled goat antimouse IgG secondary antibodies (Santa Cruz Biotechnology) (diluted to 1 : 5000) at 37°C in the dark. After washing with PBS, the cells were finally mounted and visualized with the aid of confocal microscopy (GeneAll, Seoul, South Korea).

5. Gene expression by quantitative real-time polymerase chain reaction (PCR)

The effect of Cs was monitored by examining differences in the differentiation marker CoL-I. Total RNA was extracted from each sample using a Ribospin device (GeneAll), and then converted to complementary DNA (cDNA) with a first-strand cDNA synthesis kit (PrimeScript RT reagent kit, Bioneer, Daejeon, South Korea). The reverse-transcriptase reaction was carried out using 2- μ g aliquots of total RNA. The sense and antisense primers were designed according to published cDNA sequences available in GenBank (NIH genetic sequence database), and β -actin was used as a standard housekeeping gene against which to normalize mRNA expression (as presented in Table 1)[9,10]. Real-time PCR products were monitored and quantified using a Sensimix SYBR[®] kit (Bioline, London, UK) in a Rotor-Gene RG-3000A[®] system (Corbett Research, Mortlake, NSW, Australia). After the real-time PCR ran, the threshold cycle (Ct) was used to determine the efficiency of different genes relative to the internal control (β -actin). The amount of mRNA in each sample was calculated using the comparative Δ Δ Ct method[9]. Each measurement was assessed in triplicate.

6. Western blot analysis

After the harvested cells were cultured, the total cellular proteins were prepared using 0.4 mL of cell lysis buffer (RIPA buffer, Cell signaling technology, Danvers, MA, USA). Equal amounts of each lysate were used for electrophoresis through a sodium dodecyl sulfate (SDS)-polyacrylamide gel in triglycine-SDS running buffer. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, which were then incubated in 5% bovine serum albumin blocking solution for 2 hours at room temperature and then overnight at 4°C in the presence of the CoL-I antibody (diluted to 1 : 200). After the membrane was washed with Tris-buffered saline (0.2% Tween-20) three times to remove antibodies, the horseradish-peroxidase-conjugated secondary antibody (1 : 10,000 dilution) was added to the membrane and incubated for 2 hours at 37°C[11]. After further washing in Tris-buffered saline (0.2% Tween-20), the PVDF membranes were visualized with an enhanced chemiluminescence reagent (Luminol, Santa Cruz Biotechnology) and exposed to MicroChemi 4.2 (DNR Bio-Imaging Systems, Jerusalem, Israel).

7. Statistical analysis

The *in vitro* cellular tests were carried out in triplicate, and the data are presented as mean and SD values. Differences were analyzed statistically using one-way analysis of variance, and the level of statistical significance was set at $p < 0.05$ or $p < 0.01$.

III. Results

1. Detection of CoL-I using direct immunofluorescence

Immunofluorescence staining revealed the presence of CoL-I in fibroblasts from the control, Tv2, Tv4, Tv6, and Tt samples (Fig. 1). CoL-I was much more prominent in the control, Tt, and Tv2 samples than in Tv4 and Tv6 samples.

Table 1. Real-time Polymerase Chain Reaction Primer Sequences for Genes coding for Type I Collagen (CoL-I)

	Sense primer	Antisense primer
CoL-I	5'-gtacatcagcccaaaccca-3'	5'-caggatcggaaccttcgctt-3'
β -actin	5'-tatcctggcctcactgtcca-3'	5'-acgcagctcagtaacagtc-3'

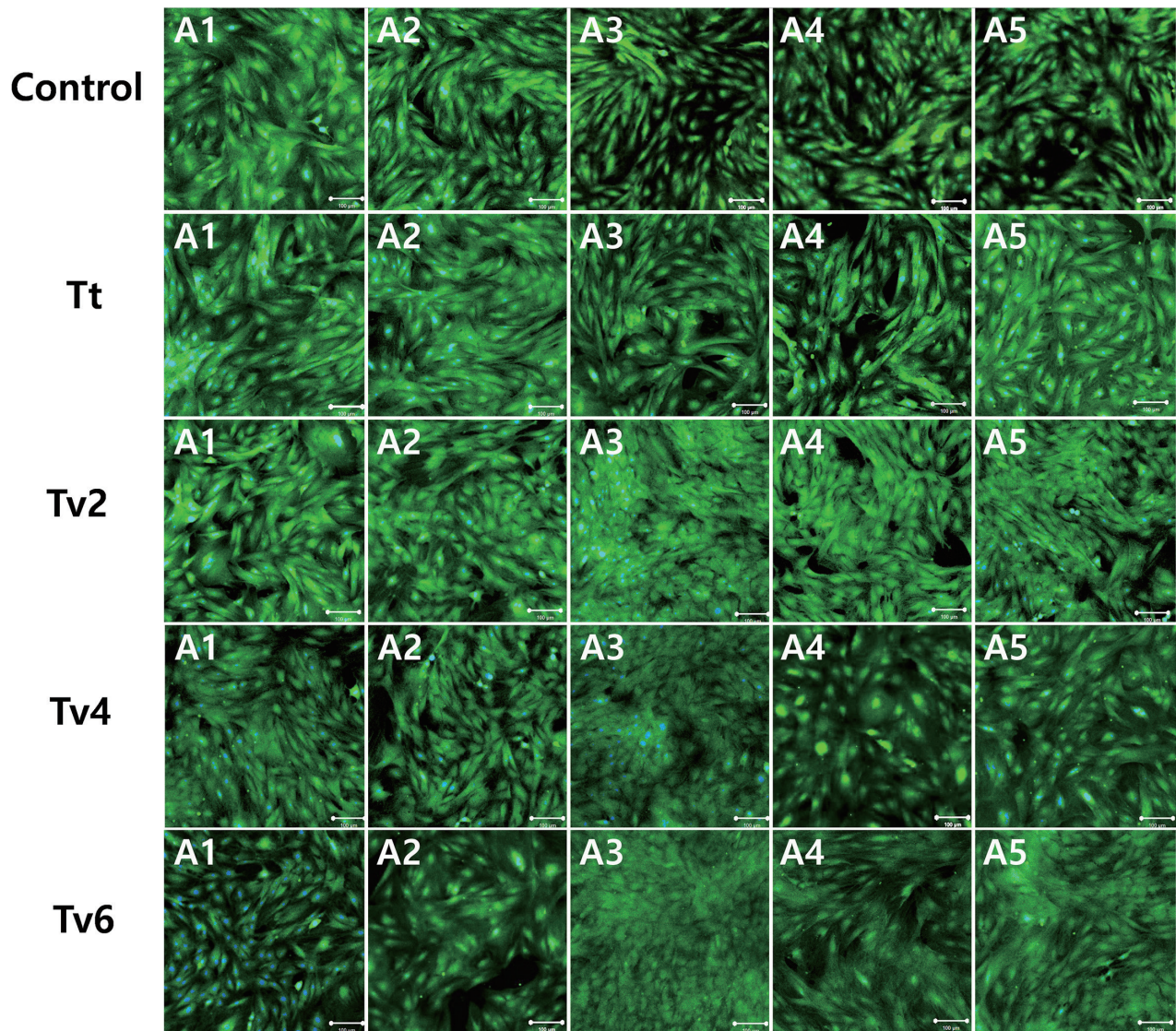


Fig. 1. Type I collagen (Col-I) appeared green in immunofluorescence staining of cultured gingival fibroblasts. Animals were designated as A1 - A5. Control : control; Tt : *in vitro* cyclosporine (Cs)-treated group; and Tv2, Tv4, and Tv6 : *in vivo* Cs-treated group at 2, 4, and 6 weeks, respectively.

2. Comparison of Col-I mRNA expression

Real-time PCR analysis was performed to quantitatively assess the mRNA expression level of Col-I in gingival fibroblasts, and the results are presented in Fig. 2. Col-I mRNA expression appeared to increase in all animals, except one, following *in vitro* Cs treatment, and the changes were not statistically significant. When Cs was administered *in vivo*, two animals exhibited considerably decreased Col-I mRNA levels after 6 weeks of Cs administration, while the other animals did not exhibit any noticeable change.

3. Analysis of Col-I protein expression by western blotting

The expression levels of Col-I protein were assessed by western blot analysis. The bands in Fig. 3 represent the expression levels of Col-I protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As shown in Fig. 4, western blot analysis revealed marked differences in the amount of Col-I protein in the Cs-treated test groups and in untreated controls. This result was similar to that obtained in *in vitro* Cs treatment.

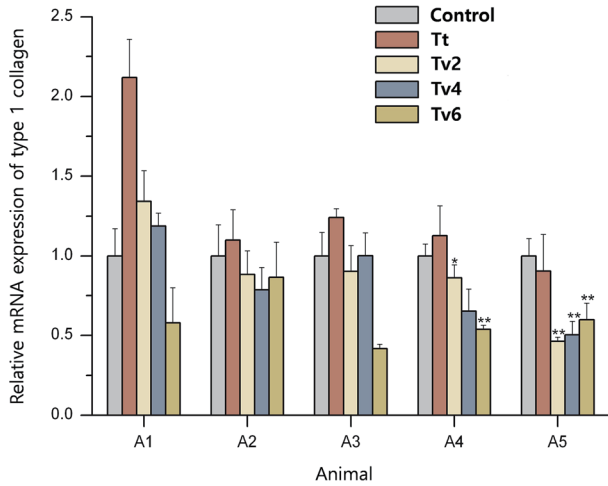


Fig. 2. Relative mRNA expression levels of CoL-I in all test groups compared with control within each animals (A1 - A5) (* : $p < 0.05$, ** : $p < 0.01$).

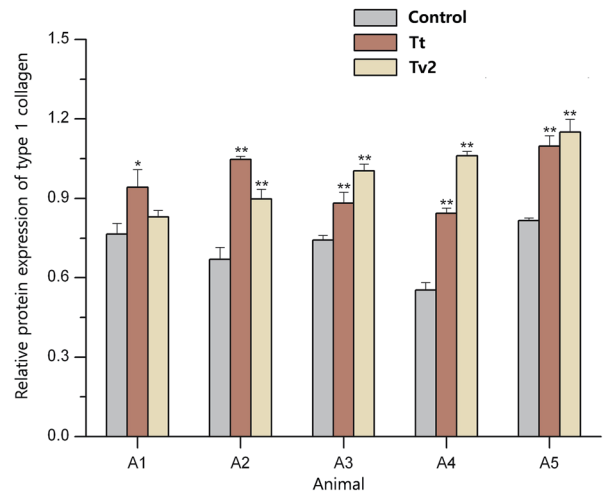


Fig. 4. Relative protein levels of CoL-I in all test groups compared with control within each animals (A1 - A5) (* : $p < 0.05$, ** : $p < 0.01$).

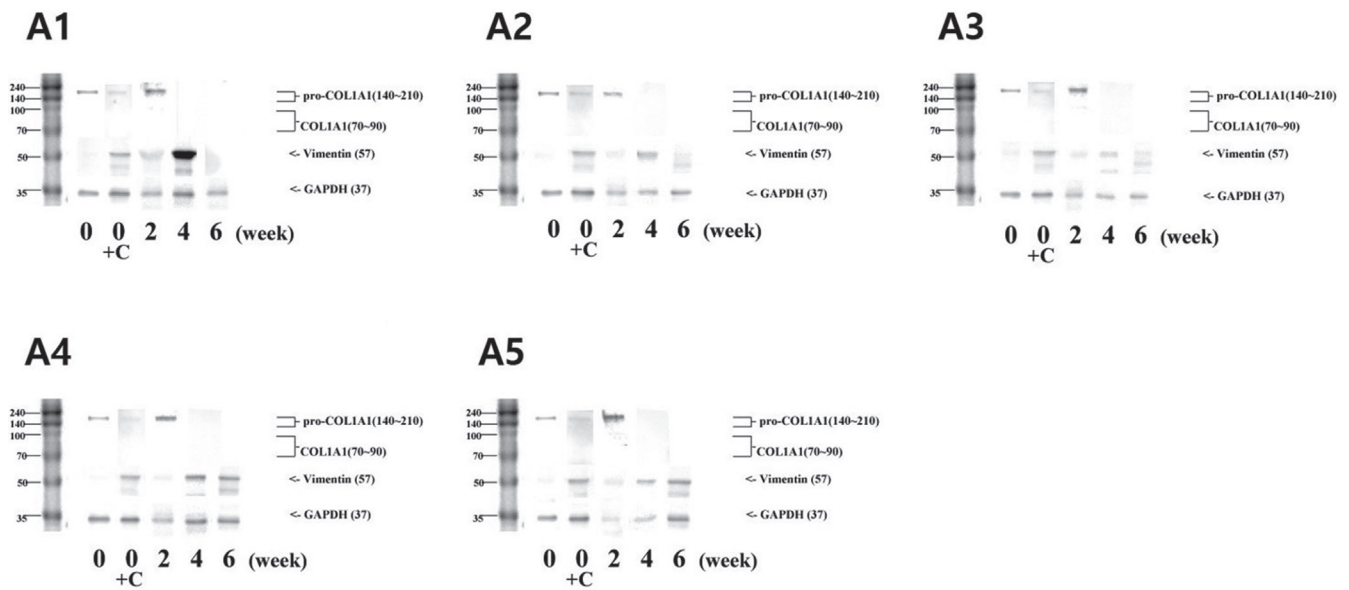


Fig. 3. Bands weighing 140-210 kDa correspond to pro-CoL-I. Bar graphs show CoL-I protein levels in each animal (A1 - A5). Data are means \pm SD of triplicate measurements (* : $p < 0.05$, ** : $p < 0.01$) compared with the control.

IV. Discussion

In patients with drug-induced GO, it is inevitable to discontinue the drug to treat GO, because GO has a high recurrence rate even with surgical treatment. Therefore, treatment

of GO remains a challenge. Considerable efforts have been made to elucidate the mechanism underlying the onset of GO following Cs administration. Although the use of Cs has decreased recently with the discovery of another immunosuppressant, FK506, it is still a widely used immunosuppressant

along with FK506. Therefore, studies on GO, an intraoral complication of Cs, are necessary for the healthy life of patients. Many studies have attempted to determine the pathogenesis of drug-induced GO in both humans and other animal models[7,12-15]; however, the mechanism remains unclear. It has been suggested that disorders of collagen metabolism in gingival connective tissue are mostly related to the pathogenesis of GO[3,4,7,8,14,16]. In previous studies, the effects of Cs were analyzed using gingival fibroblasts harvested from different individuals in the control and test groups. Therefore, these studies did not consider the effects of factors other than CoL-I in different individuals. Since all individuals do not share the same biologic conditions, it is necessary to control all factors other than the factor selected for the study when evaluating drug effects. Tipton *et al.*[17] demonstrated variations among individuals and heterogeneity of human gingival fibroblasts and found that fibroblasts from different individuals varied markedly in collagenolysis and production of proteins in response to Cs treatment. Furthermore, the genetically determined capacity of the host to cope with the administered Cs, the responsiveness of gingival tissue to the drug, and periodontal conditions increase the difficulty of examining the etiology of Cs-induced GO. In the present study, cultures of gingival fibroblasts were harvested from the same rat before and after Cs treatment to exclude inter-individual variations. To overcome the limitations in the experimental design of previous studies, the present study analyzed the changes in CoL-I in the same individual by assigning specimens harvested from rats prior to Cs administration to the control group and specimens harvested from the same rats after Cs administration to the test group. Our data from gene and protein expression analyses showed a considerable gap among individuals, indicating variations. In addition, the study compared the results of *in vivo* Cs treatment against the results of *in vitro* Cs treatment applied on some gingival fibroblasts in the control group prior to *in vivo* Cs treatment. This step was designed to determine whether GO incidence could be predicted with only the results of laboratory test performed on gingival fibroblasts prior to drug administration. The results of the present study showed that CoL-I protein expression level increased after both *in vitro* and *in vivo* Cs treatment.

Some *in vitro* studies have revealed an increase or decrease in CoL-I mRNA expression levels[8,18]. This discrepancy may have originated from the culture conditions and indicate the need to concomitantly conduct *in vivo* studies. Our real-time

PCR analysis clearly showed that the mRNA expression levels of CoL-I were inconsistent between *in vitro* and *in vivo* Cs administration, compared with the control. In contrast, CoL-I protein analysis in our study demonstrated upregulated CoL-I expression in all subjects in both *in vitro* and *in vivo* Cs treatment. A marked increase in CoL-I expression was observed approximately 2 weeks after *in vivo* treatment, and it corresponded to the increase observed after *in vitro* treatment. The data on CoL-I protein expression confirmed its tendency to increase after Cs treatment regardless of mRNA expression, as shown previously[18]. This result suggests that the regulation of CoL-I protein expression may primarily occur at the post-transcriptional level. During soft tissue fibrosis, CoL-I protein expression is highly upregulated both transcriptionally and post-transcriptionally[19-21]. Transcription alone is not sufficient to regulate the expression of CoL-I[22]. The production of CoL-I can be increased several hundred-fold in wound healing or in pathological fibrosis. This increase is principally due to post-transcriptional regulation, followed by increased half-life and stability of mRNAs, and transcription rate of collagen genes[23-25]. These studies are in agreement with our study showing that CoL-I protein expression considerably increased in two animals(Fig. 3), with significantly decreased mRNA expression levels 2 weeks after Cs administration(Fig. 2).

We found that Cs treatment had no specific effect after 4 weeks of administration. This finding may be supported by studies on the pathogenesis of drug-induced GO in a rat model[25-27]. When Cs was administered to 4-week-old rats, macroscopic GO was detected as early as 3 weeks, and the severity of overgrowth increased slightly, reaching a plateau by approximately 6 weeks[28]. In the present study, 5-week-old rats were used, and the induction of GO was maximal after 2 weeks of Cs administration. Longer treatment periods of up to 10 weeks did not result in further GO[26]. No noticeable bands were observed in western blot analysis for CoL-I protein after 4 and 6 weeks of Cs administration in the present study.

V. Conclusion

We performed an experiment that considered inter-individual differences and controlled factors other than CoL-I. It cannot be concluded that the protein level of CoL-I is a marker for GO based on the results of this experiment alone. Nevertheless, this experimental design that compared the results at 2, 4, and 6 weeks after *in vitro* and *in vivo* Cs administration

is useful, because if the response of markers, such as CoL-I, in preoperative *in vitro* setting corresponds to the response after *in vivo* treatment in the same individual, then predictions on GO can be made prior to *in vivo* treatment. It is suggested that markers other than CoL-I, which were used in previous studies, should be investigated using the experimental design of the present study. In future, studies on human gingival fibroblast are warranted.

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국문초록

소아에서 치은 과증식에 대한 cyclosporine의 효과를 연구하는 새로운 방법

한금아¹ 임상교수 · 김종수² 교수

¹ 단국대학교 치과대학 세종치과병원 치주과

² 단국대학교 치과대학 소아치과학교실

소아에서 사이클로스포린에 의해 유발되는 치은과증식의 병인을 규명하기 위한 이전의 연구에서는, 개체 간의 차이를 유발할 수 있는 요인을 완전히 배제하지 않았다. 본 연구는 개체 별로 생물학적 변이의 영향을 통제한 실험 모델에 의해 사이클로스포린이 1형 교원질의 대사에 미치는 영향을 알아보았다. 5주령의 수컷 Sprague-Dawley rats 5마리에 6주 동안 사이클로스포린을 위장으로 투여하였다. 사이클로스포린 투여 전과 투여 후 2, 4, 6주째에 쥐의 하악구치부에서 치은을 채취하였다. 20개의 치은표본으로부터 치은섬유모세포를 추출하였다. 개체 별로 사이클로스포린을 투여하기 전 채취한 치은섬유모세포의 반은 대조군, 나머지 반은 실험실 내 약물 처리한 실험군(Tt)으로 하였다. 사이클로스포린 투여 후 2, 4, 6주째 채취한 치은섬유모세포는 각각의 실험군으로서 T2, T4, T6으로 명명하였다. 면역형광법을 시행하여 모든 치은모세포 내의 1형 교원질의 존재를 확인하였다. 각 개체 별로 대조군과 실험군에서 1형 교원질의 유전자와 단백질 발현의 변화를 분석하기 위해서 각각 Real-time polymerase chain reaction과 Western blotting을 시행하였다. 사이클로스포린을 처리하기 전과 후를 한 개체내에서 비교하여 1형 교원질의 변화를 평가하였다. 대조군과 실험군에서 1형 교원질의 유전자 발현에서는 유의한 차이가 없었다. 모든 개체에서 실험실 내 사이클로스포린 처리한 치은섬유모세포의 1형 교원질의 단백질 발현이 증가한 경우, 사이클로스포린을 투여한 후 채취한 치은섬유모세포에서도 1형 교원질의 단백질 발현이 증가하였다. 본 연구에서 시행한 개체별로 발생할 수 있는 생물학적 변이를 모두 통제한 실험 방법은 유용하였다. 1형 교원질 이외에 다른 요인들에 대한 후속 연구 및 인간에서의 심도 있는 연구가 필요하다.