EFFECT OF GLYCYRRHETINIC ACID ON CYCLOSPORIN A-INDUCED CELL ACTIVITY OF CULTURED HUMAN GINGIVAL FIBROBLATS AND MOUSE 3T3 CELLS *IN VITRO*

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I. INTRODUCTION

Cyclosporin(Cs) is a hydrophobic, cyclical polypeptide derived from the metabolic products of two fungal species, *Trichodema polysporum* and *Cylindrocarpon lucid* compound had an inhibitory effect on lymphocyte proliferation[1]. This is a very immunosuppresant acting mainly on T-lymphocytes by blocking production responsiveness to, interleukin-2 (IL-2)[2-3]. In addition to the direct effect on T-lymphocytes, cyclosporin indirectly alters monocyte function by suppressing production of a variety of T-cell lymphokines such as gamma-interferon, macrophage chemotactic factor.[4] As a result,interleukin-1 production by monocytes is inhibited and this has the potential to affect not only inflammatory cells but also connective tissue cells[7-8].

Although Cs appears to be uniformly beneficial in the treatment of a variety of disorders, its use may result in a number of adverse reactions. The most conmonly observed side effects are nephrotoxicity, hepatotoxicity, hirsutism, mild tremor, gastrointestinal disturbances, and gingival overgrowth[10,11].

The reported incidence of gingival overgrowth in Cs-treated patients may approach 30% [11-12] and is clinically similar to the effect seen with the anticonvulsant drug phenytoin[13,14] and the calcium channel antagonist nifedipine [15]. Gingival overgrowth is first seen between 4 and 6 weeks after commencement of drug therapy and the clinical presentation may vary from an inflamed, hemorrhagic and edematous gingiva to a tissue which is fibrous with minimal inflammation[16].

Recently, fibroblasts isolated from overgrowth gingiva from patients receiving Cs were found to possess different metabolic and proliferative activities when compared with fibroblasts from healthy gingival sites from the same donor[17]. These findings raise the possibility that, in these lesion, Cs affects not only T cells but also the finbroblastic cells responsible for the production of the extracellular matrix. Whether this occurs via the direct action of Cs on fibroblasts, or reflects a secondary response due to immunomodulation, is not clear.

Glycyrrhizin(GL), a main component of liquorice(*Glycyrrhiza glabra* L.), is ingested

orally as a sweetener or as a component in oriental medicine. GL has a steroid-like action [18] and an anti-viral activity[19]. GL is metabolized to glycyrhetinic acid(GA), and then 3-epi-18β-glycyrrhetic acid via 3-keto-18β-glycyrrhetic acid(3-ketoGA), by human intestinal bacteria[20]. GA shows pronounced anti-inflammatory activity[21,22]. Early reports indicated that liquorice extract had a deoxycorticosterone-like activity[23] and that glycyrrhizin had a corticoid-like action by inhibiting the metabolism of corticoids. Kumagai[18,24-26] reported its anti-estrogenic action. A study[27] has shown that glycyrrhetinic acid on cultured melanoma cells caused growth inhibition and stimulate melanogenesis.

The purpose is to examine the effect of cyclosporin on gingival fibroblasts and analyze the possible role of glycyrrhetinic acid in response to co-incubation with cyclosporin and glycyrrhetinic acid.

II. MATERIALS AND METHODS

Fibroblast culture

Fibroblasts derived from human gingiva were isolated from interproximal premolar or molar gingival papilla of clinically healthy tissue(firm, non-edematous and pink tissue which does not bleed upon probing) from adults. Normal human gihgival cells(NHGF) were isolated from healthy dornors. These tissue explants were placed in 60mm tissue culture plates containing culture medium composed of : minimum essential medium(α-MEM) with ribonucleoside and deoxyribonucleoside containing 10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25μg/ml amphotericin B as fungizone ; and 10 % heat-inactivated fetal bovine serum(FBS). The cells were incubated at 37℃ in humidified atmosphere of 5% CO_2 and 95% air. The medium was replaced 2 or 3 days until sufficient cell proliferation was evident. Trypsin/EDTA was used to detach the cells, which were transferred into 60mm dishes for continued growth, These cells were maintained in culture medium with 10% FBS and passed as needed for experimentation. Cells were used between the 4th and 8th passages.

Cyclosporin preparation

Stock solution of cyclosporin A (CsA) was prepared at a final concentration of 1mg/ml, filter sterilized, and then diluted with culture medium to make a starting concentration of 10^{-6}g/ml. The chemical structures of material is shown in Fig. 1A. The solvent containing 10% ethanol and 90% olive oil was used to prepare the stock solutions of CsA.

Glycyrrhetinic acid preparation

Glycyrrhetinic acid(GA, Sigma) was used in the present experiments. The structures of GA and its related compound, glycyrrhizin (GL), are shown in Fig. 1B, GA was first dissolved in a small volume of ethanol and diluted with culture medium to desired concentrations.

Observation of cell morphology

Cells were plated in 35/mm dishes with 1ml of the media containing different glycyrrhetinic acid(GA) or cyclosporin A(CsA) concentration. The concentration of fetal bovine serum(FBS) in the experimental media was 10 %. At the termination of the experiments on 4 day, the medium was removed from the dishes and cell monolayer was rinsed with phosphate-buffered saline(PBS ; 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, and 0.2g KH_2PO_4 in 1000ml H_2O, pH 7.2). The PBS solution containing 50% methanol(PM solution) was poured into the dishes and half volume of PM

solution was discarded and then replaced with fresh methanol for 10 min at room temperature. All of solution were discarded and replaced with fresh anhydrous methanol. The methanol was discarded after rince of monolayer. Then, the dishes may be dried and stored or stained directly. Neat Giemsa stain, 2ml per 25mm petri dish, was added to cover the entire monolayer. After 2min, the stain was diluted with 8ml water and agitated gently for further 2 min. Vigorus washing in running tap water was continued until any pink cloudy background stain(precipitate) appeared. After pouring off water, and rinsing in deionized water, the samples were examined under inverted-microscope.

Assay of cell activity

The endpoint of a microtitration assay is usually an estimate of cell number. While this can be direct by cell counts or indirect methods such as isotope incorporation, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide(MTT, Sigma) reduction as a cell viability measurement is now widely chosen as the optimal endpoint. MTT was removed, and the water-insoluble MTT-formazan crystals dissolved in dimethyl sulfoxide(DMSO). A buffer was added to adjust the final pH and absorbance was recorded in an ELISA plate reader.

DNA isolation by salt extraction

Cells were plated in 60/mm Nunc dishes in media containing 100μg/ml GA concentration. After incubation for 6 hrs and 18hrs,cells were harvested, lysed with 500μl of 10% SDS, mixed gently and treated with 100μl proteinase K(10 mg/ml). The cells were incubated at 37℃ for overnight or at 50℃ for 2 hrs and added with 6M NaCl in a 3/1 ratio(sample/6M NaCl). The cells were centrifuged and the supernatants were transferred to new tube and added with ethanol in a 2/1, ratio(ethanol/supernatant). The tubes were incubated for 10 minutes, and then mixed slowly until a solid white mass appeared. The solution was centrifuged and the supernatants were discarded. The DNA pellet was dissolved in 10mM Tris-HCl(pH 8.0) containing 1 mM EDTA. DNA samples were analyzed by 2% agrose gel electrophoresis.

III. RESULTS

Cell morphology

Control human gingival fibroblast cells in culture exhibited normal stretched cytoplasmic processes and formed monolayer with a few round cells(Fig. 1A, 1B). Cyclosporin A treatment increased number of fibroblasts, often with enlarged ovoid vesicular nuclei(Fig. 1C−1F). Cells attached after treatment with 100μg/ml GA for 18 hr were observed to have more extended cytoplasmic processes than untreated cells(Fig. 3D). However, the cells treated with high concentration of GA for long period showed round morphology(data not shown), detached from monolayer and floated into growth media.

These floating cells resulted decreased number of attached cells(Fig. 2D). Cells treated with CsA plus GA showed enlarged ovoid vesicular nuclei than cells treated with CsA only, and cells treated with high concentration of GA became rounding and floationg(Fig. 3C, 3D).

Effect of cyclosporin A on the activity of human gingival fibroblasts and mouse 3T3 cells

The growth activity of cyclosporin A at concetrations in the range 1 ~100ng/ml was determined after incubation for 1-5 days. With

Fig. 1. Microscope of human gingival fibroblast cells cultured in the absence(A and B) or presence (C−F) of cyclosporin A. Cells were grown for 4 days in control medium (A and B) or in medium containig 1ng/ml(C and D) and 10ng/ml(E and F) cyclosporin A.

Fig. 2. Microscope of human gingival fibroblst cells cultured in the absence(A, C) or presence(B, D) of 100μg/ml glycyrrhetinic acid. Cells were grown for 6 hr(A, B) or 18 hr(C, D).

Fig. 3. Microscope of fibroblast cells treated for 4 days with 1ng/ml cyclosporin A and various concentrations of glycyrrhetinic acid(A, 0 μg/ml ; B, 20 μg/ml ; C, 50 μg/ml ; D, 100μg/ml).

Table 1. Effect of various concentrations of cyclosporin A (CsA) treated for different time periods on the activity of human gingival fibroblast cells as determined by MTT assay. The results represent the mean% ± S.D. of MTT activity in CsA treated cells relative to that in control.

CsA (ng/ml)	CsA treatment periods(day)			
	1	3	4	5
1	123.21± 5.01*	113.09± 0.38*	113.74± 6.62*	98.62± 4.89
10	121.11± 7.62*	106.09± 4.64	119.03± 12.49*	99.37± 4.15
100	125.90± 7.88*	108.81± 5.65	117.84± 8.67*	94.72± 5.74

* : Significantly different from control(<0.05)

Table 2. Effect of various concentrations of cyclosporin A treated for different time periods on the activity of mouse 3T3 cells as determined by MTT assay. The results represent the mean% ± S.D. of MTT activity in CsA treated cells relative to that in control.

CsA (ng/ml)	CsA treatment periods(day)			
	1	3	4	5
1	92.84± 11.40	97.44± 5.94	121.50± 2.60*@	79.98± 6.57*
10	81.54± 5.11	103.56± 5.62	112.03± 9.45*@	92.33± 8.72#
100	86.49± 7.33	106.16± 2.74	99.90± 9.40	85.53± 15.76*

* : Significantly different from control(<0.05)

: Significantly different from 1ng/ml cycloporin A treated group(<0.05)

@ : Significantly different from 100ng/ml cycloporin A treated group(<0.05)

increating concentations of cyclosporin A (CsA), increased cell activity was oberved in human gingival fibroblast cells(Table 1) and mouse 3T3 cells(Table 2) compared with the control culture in the absence of cyclosporin A. As shown in Table 1, the activity of cells treated with cyclosporin A at concentrations of 1, 100ng/ml for 4 days increased significantly compared with that in control human gingival fibroblast. After 4 days in clulture, cells reached confluency and exhibited inhibition effects.

Effect of glycyrrhetinic acid on the activity of human gingival fibroblastand 3T3 cells

The growth inhibitory effect of GA at concentrations of 5−100 μg/ml was determined after 1−3 days incubation. GA inhibited the growth of mouse 3T3 cells in a dose-dependent manner and caused complete inhibition at concentrations over 25 μg/ml(Fig. 4A, 4B), while human gingival fibroblast did not inhibited cell growth even at concentrations over 50 μg/ml(Fig. 4C).

Effect of glycyrrhetinic acid with cyclosporin A(1ng/ml) on the activity of human gingival fibroblasts

GA was added in a range of concentrations from 10−100μg/ml in the presence of CsA. At these concentrations, GA showed an inhibitory effect on cell activity, compared with cells exposed to CsA only. The inhibitory effect was remarkable at 100 μg/ml GA(Fig. 5A). CsA

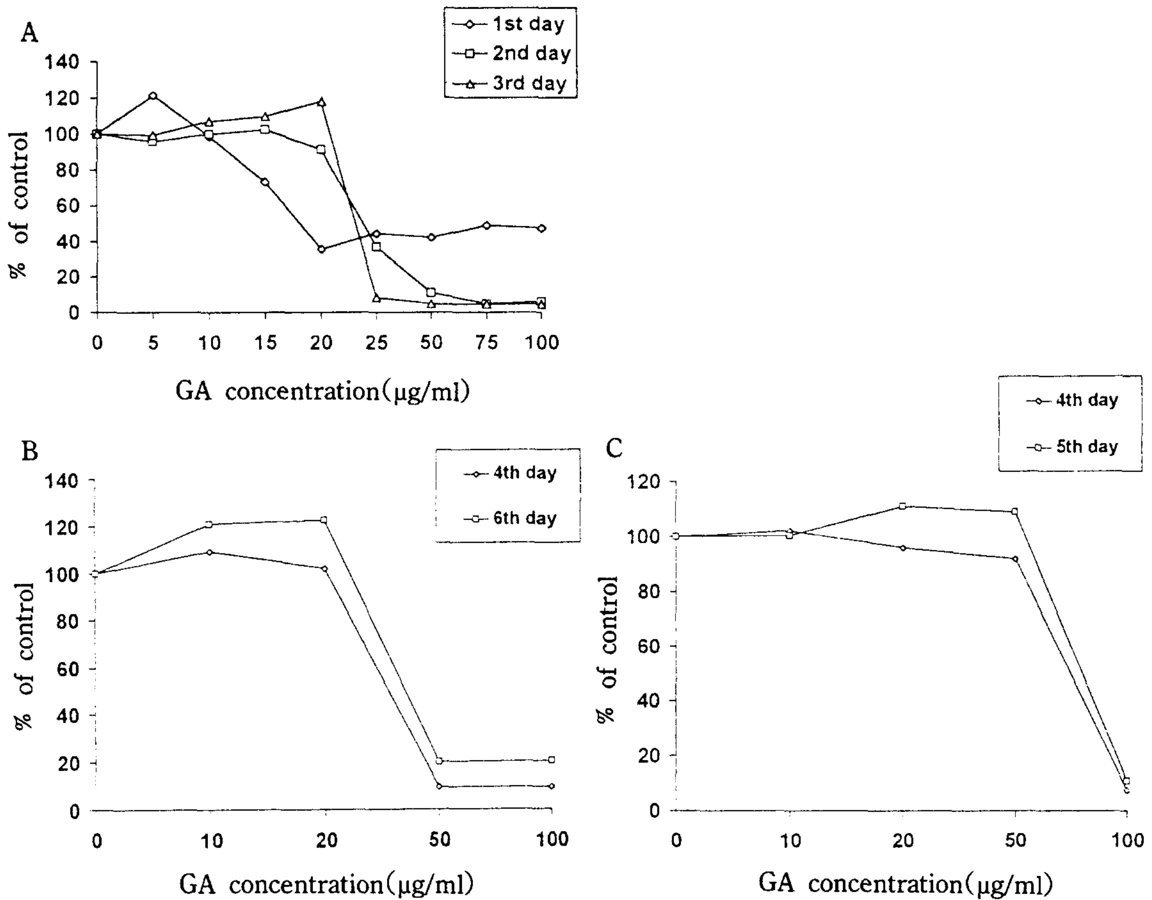

Fig. 4. Effect of various concentrations of glycyrrhetinic acid treated for different time periods on the activity of mouse 3T3 cells (Panel A and B) or human gingival fibroblast cells(Panel C) ans determined by MTT assay.

plus GA showed an inhibitory effect on cell activity, compared with cells exposed to GA only. The synergistic effect was remarkable at 50 μg/ml GA plus 1ng/ml CsA.

Effect of glycyrrhetinic acid with cyclosporin A(10ng/ml) on the activity of human gingival fibroblast

In order to verify that GA inhibits fibroblasts proliferation, GA was added in a range of concentrations from 10−100 μg/ml in the presence of CsA. Addition of GA to the human gingival fibroblasts containing CsA caused a change to the levels of cell activity(Fig. 5B). At a concentration of 100μg/ml, GA showed a strong inhibitory effect on cell activity.

Effect of glycyrrhetinic acid with cyclosporin A(10ng/ml) and 1ng/ml) on the activity of mouse 3T3 cells

The cell activity in CsA-containing medium was inhibited by glycyrrhetinic acid(at 50μg /ml over, $P<0.05$). But, 10 and 20 μg/ml-GA

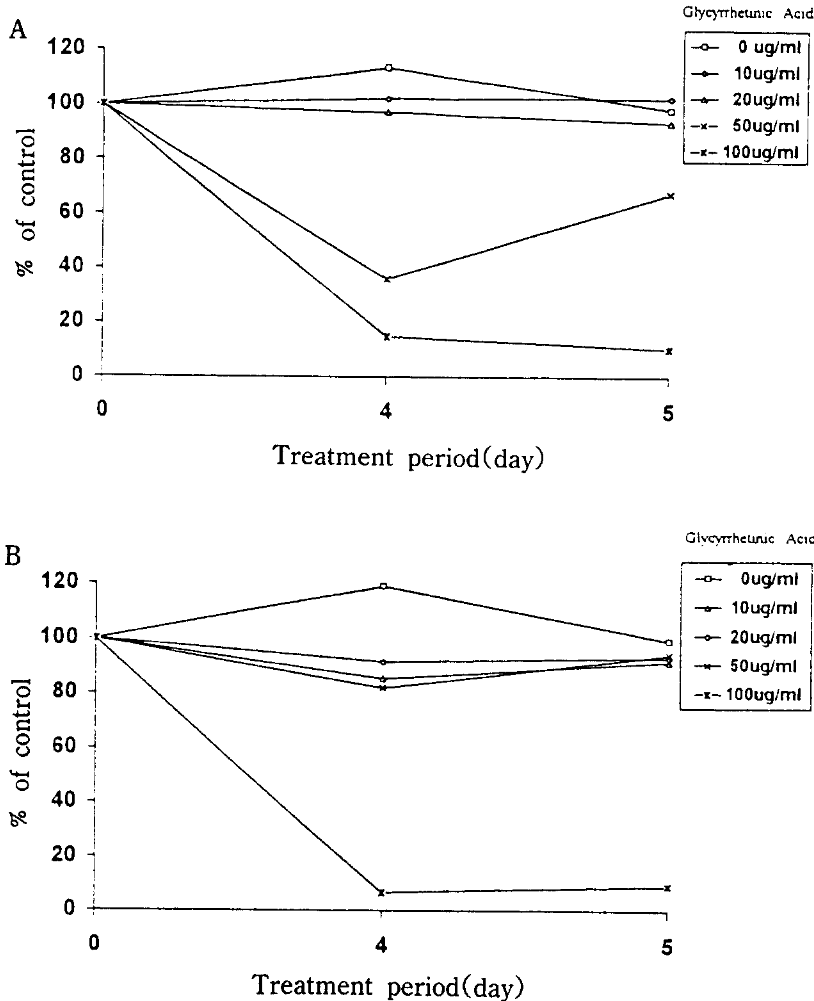

Fig. 5. Effect of combined treatment of fixed concentration of cyclosporin A and various concentrations of glycyrrhetinic acid on human gingival fibroblast activity. Human gingival fibroblast cells were treated with various concentrations of glycyrrhetinic acid in the presence of 1ng/ml(Panel A) or 10ng/ml(Panel B) cyclosporin a for different time periods.

treated cells in CsA containing medium showed no inhibiton effect on cell activty. In the presence of 50 and 100μg/ml of glycyrrhetinic acid, the activity of the CsA-treated cells was inhibited almost completely(Fig. 6A, 6B). In the presence of 50 μg/ml of GA, plus GA showed an additive effect on cell atcivity, compared to cells exposed to GA only.

Effect of glycyrrhetinic acid on DNA fragmentation of human gingival fibroblast and 3T3 cells

Fig, 7 clearly demonstrates that DNA fragmentation was occurring when cells were stimulated with glycyrrhetinic acid. To examine time course of DNA fragmentation, 3T3 cells

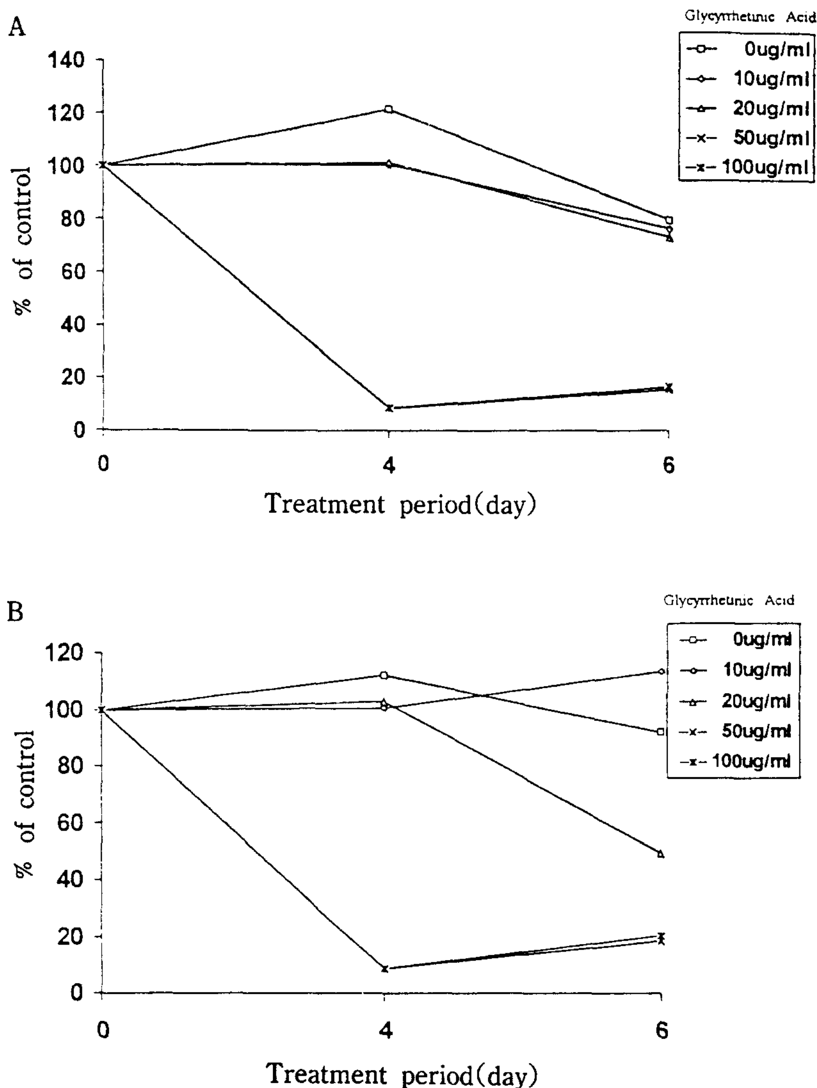

Fig. 6. Effect of combined treatment of fixed concentration of cyclosporin A and various concentrations of glycyrrhetinic acid on 3T3 cell activity. 3T3 cells were treated with various concentrations of glycyrrhetinic acid in the presence of 1ng/ml(Panel A) or 10 ng/ml(Panel B) cyclosporin A for different time periods.

were cultured with GA 100μg/ml for 6 hrs and 18 hrs. At 6 hr after GA treatment, DNA fragmentation in 100μg/ml GA treated cells was not greatly different from that in control. At 18 hr, DNA fragmentation reached a maximal level and, to a lesser degree, by 100μg/ml of GA in human gingival fibroblast cells.

Ⅳ. DISCUSSION

Cyclosporin is a selective immunosuppressant that has a variety of applications in medical practice. Like phenytoin and the calcium channel blockers, the drug is associated with gingival overgrowth. The phamacodynamics

Fig. 7. Agrose gel electrophoresis of DNA extracted from 3T3 cells(Panel A) and human gingival fibroblast(Panel B). Cells were cultured for 6 hr(A : lane 1 and 2) or 18 hrs(A : lane 3 and 4, B : lane 1 and 2) in medium without(A : lane 1 and 3, B : lane 1) or with(A : lane 2 and 4, B : lane 2) 100μg/ml glycyrrhetinic acid. DNA was extracted from each group and electrophoresed on 2% agrose gel. M dentotes λ/Hind III and $\phi \times 174$/Hae III marker DNA.

of cyclosporin mainly involve the T-cell response and the role of these cells in graft rejection were summarised as follows : (a) recognition of antigen(graft tissue) as foreign material, (b) processing of antigen by macrophages with the subsequent production and release from the cell of interleukin 1(IL−1), (c)IL−1 activation of precursor cytotoxic T−lymphocytes which acquire receptors for interleukin 2(IL−2), (d) activation of T−helper lymphocytes with the production and release of IL−2, which is accentuated by IL−2, (e) the clonal amplification of activated cytotoxic T-lymphocytes which cause cell mediated lysis and graft rejection, (f) the activation of suppressor T-lymphocytes which can modulate these response.

Cyclosporin inhibits many of the stages outlined above, acting at both a cellular and molecular level. Specifically, the drug inhibits IL−2 synthesis at concentrations of between 10−20 ng/ml. At a higher concentration(100 ng/ml), cyclosporin inhibits the ability of cytotoxic T-lymphocytes to respond to IL-2. The mechanism of this inhibition is uncertain, but may be due to the drug blocking the induction of IL−2 receptors on these cells[28].

Cyclosporin A has the capacity to stimulate fibroblast proliferation directly since fibroblasts derived from normal healthy gingiva were shown to have significantly increased DNA synthesis. The fact that cells derived from CsA affected tissue did not display significant increased proliferation in response to Cs in culture supports the finding that the CsA lesion does not represent a cellular hyperplastic response[29]. The effect of CsA on gingival fibroblast proliferation was also monitored using a direct measure of cell numbers. Although maximum proliferation occurred around 2 and 3 days after treatment, the cells cultured in the presence of CsA showed more significant increase in growth than the control cultures 1 and 2 days[30](Table 1).

Androgens are metabolized actively in gingival tissues. Rappaport[31] found that testos-

terone metabolism occurred to a greater extent in healthy gingiva of men than women, due mainly to increased formation of 5α-dihydrotestosterone(5α-DHT), the biologically-active androgen, which stimulates biosynthetic activity in fibroblasts. In inflamed gingival tissue, however, the extent of metabolism was similar in males and females. Consistent features in both inflamed and hyperplastic gingival tissue were shown to be marked increases in the formation of 5α-DHT and 4-androstenedione[32] and a corresponding increase in the number of gingival receptors for 5α-DHT[33]. Previous studies have shown that significant levels of androgen metabolism occur in gingival hyperplasia induced by cyclosporin A, compared with of non-hyperplastic controls. Inflamed gingival tissue has also been found to convert testosterone to the biologically-active metabolite, 5α-DHT in significant quantities[32,33]. This metabolite has a stimulatory effect on the synthetic activity of fibroblasts. Therefore, baseline androgen metabolism in the gigival tissue may have some bearing on the incidence of drug-induced hyperplasias, in the presence of plaque-associated gingival inflammation.

Oral administration of glycyrrhetinic acid was shown to decrease *in vitro* basal testosterone production in Leydig cells by luteinizing hormone stimulation. Glycyrrhizin and glycyrrhetinic acid caused a significant decrease in testosterone production with an accumulation of 17α-hydroxyprogesterone. Glycyrrhetinic acid was shown to inhibit the conversion of androstenedione to testosterone, indicating that these compounds inhibit the activity of 17β-hydroxystroid dehydrogenase[34]. The inhibitory effect of glycyrrhetinic acid was far more potent than that of glycyrrhizin, as judged from the present result showing 90% inhibition at 50 μg/ml glycrrhetinic acid(Fig. 4A) compared with the reported 90% inhibition at 200μg/ml glycyrrhizin[27]. And cells treated with 100μg/ml glycyrrhetinic acid showed disappearence of cytoplasmic processes and exhibited rounding shape(Fig. 3D). But, growth inhibition of glycyrrhetinic acid was interpreted as a result of cytostatic but not cytotoxic effects of glycyrrhetinic acid by examinations of the cell viability and plating efficiency of B16 melanoma cells treated with glycyrrhetinic acid[27].

Programmed cell death(PCD), or apoptosis, is a physiological mechanism of cell deletion that differs morphologically and biochemically from necrosis[35,37]. PCD is involed in normal tissue turnover during embryogenesis and adult life, including thymus involution[36,38], control of hemopoietic stem cell growth[39-41], and the negative thymic selection of the T-cell repertoire[42]. The mechanism of PCD follows incomplete signal transduction, such as increase of isolated Ca^{2+} or cAMP[43-46], and also requires initiation of protein synthesis and is associated with endogenous endonclease activation. Biochemical mechanism of the process is characterized by regular fragmentation of the entire celular DNA into oligonucleosome fragments, all of which are multiples of a 20 base pair unit[43-46], when cells were incubated with 100μg/ml glycyrrhetinic acid for 18 hr, DNA fragmentation exhibited(Fig. 7).

In our experiment, when glycyrrhetinic acid was added in a range of concentrations from 10−100μg/ml in the presence of cyclosporin A, cell activity was inhibited significantly, compared with that in cyclosporin A-only treated cell. Paticularly, at glycyrrhetinic acid 100μg/ml, the cell activity was inhibited completely (Fig. 5−6). A synergitic effect was noted when the cell activity of gingival fibroblasts was assessd in response to co-incubation with 1ng/ml cyclosporin A and 50μg/ml glycyrrhe-

tinic acid(Fig. 5A) and additive effect was noted when the cell activity of 3T3 cells was assessed in response to co-incubation with cyclosporin A and glycyrrhetinic acid(Fig. 6).

These results suggest that effect of glycyrrhetinic acid with cyclosporin A on the activity of human gingival fibroblast have a synergistic effect.

V. CONCLUSION

1. Cells treated with CsA plus GA showed enlarged ovoid vesicular nuclei than cells treated with CsA only, and cells treated with high concentraton of GA became rounding and floating.
2. The activity of cells treated with cyclosporin A at concentrations of 1~100ng/ml for 4 days increased significantly compared with that in control human gingival fibroblast.
3. Glycyrrhetinic acid inhibited the growth of 3T3 cells in a dose-dependent manner and caused complete inhibition at concentrations over 25μg/ml.
4. In human gingival fibroblast, the synergistic effect was remarkable at 50μg/ml glycyrrhetinic acid plus 1ng/ml cyclosporin A.
5. In the presence of 50μg/ml of glycyrrhetinic acid, cycloporin A plus glycyrrhetinic acid showed an additive effect on the activity of 3T3 cells.
6. When 3T3 cells were cultured with 100 μg/ml glycyrrhetinic acid for 18 hrs, DNA fragmentation reached a maximal level.

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국문초록

시험관 내에서 Glycyrrhetinic acid가 배양 치은 섬유모세포와 마우스 3T3 세포의 Cyclosporin A 유도 활성 세포에 미치는 영향

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배양한 치은섬유아세포와 3T3 세포에서, glycyrrhetinic acid가 cyclosporin A를 처리 세포의 활성에 미치는 영향을 알아보기 위해서 MTT 방법을 이용하여 측정하였다. Cyclosporin A는 1~10ng/ml의 농도에서 인체 치은 섬유아세포와 마우스 3T3 세포 활성을 증가시켰으며, glycyrrhetinic acid는 농도와 비례하게 마우스 3T3 세포의 성장을 억제시켰다. 특히 25μg/ml 이상의 농도에서는 현저하게 억제시킴을 관찰할 수 있었다. 반면, 인체 치은 섬유아세포에서는 50 μg/ml 이상의 농도에서 조차도 성장을 억제 시키지 않았다. 또한, 마우스 3T3와 인체 치은 섬유아세포에서 일정한 cyclosporin A 농도에 1－100μg/ml의 다양한 농도로 glycyrrhetinic acid를 첨가하였을 때, cyclosporin A 단독으로 처리한 세포에 비하여 유의성 있게 세포의 활성을 억제시켰으며, 특히, 100μg/ml의 glycyrrhetinic acid를 첨가하였을 경우 세포활성을 현저하게 억제시켰다.

3T3 세포에 cyclosporin A와 50μg/ml의 glycyrrhetinic acid를 함께 처리한 경우 glycyrrhetinic acid단독처리한 군에 비하여 세포활성에 첨가 효과를 보였으며, 인체 치은 섬유아세포에서는 같이 처리한 경우 glycyrrhetinic acid단독 처리에 비하여 뚜렷한 억제 효과를 나타내었다. 이러한 상승효과는 1ng/ml의 cyclosporin A와 50μg/ml glycyrrhetinic acid를 같이 처리한 군에서 가장 뚜렷하게 관찰되었다.

이러한 결과로, 인체 치은 섬유아세포에 cyclosporin A와 glycyrrhetinic acid를 동에 처리하였을 경우 세포의 활성에 상승 효과가 있음을 알 수 있다.