

Effect of glycyrrhetic acid on the viability of human gingival fibroblasts

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I. Introduction

Some therapeutic agents and medicines such as phenytoin (PHT), nifedipine and cyclosporine may lead to adverse effects causing pathologic changes in tissue, especially on the gingivae¹⁻⁸). The common histological finding is a marked gingival hyperplasia, with proliferation of gingival fibroblasts and an increase of collagen fibers in connective tissue. PHT-induced human gingival overgrowth may be genetically programmed for increased synthesis of collagen⁹). PHT-induced gingival overgrowth was preceded by the inflammation of gingivae¹⁰⁻¹¹). Previous studies reported that PHT, nifedipine and cyclosporine increased the cell activity of human gingival fibroblasts¹²⁻¹⁷).

Some differentiation factors such as glycyrrhetic acid (GA), retinoic acid, ursolic

acid and oleanolic acid inhibited¹⁸⁻²⁵). Previous studies reported that GA inhibited intercellular communication between human fibroblasts²⁶⁻²⁷).

Glycyrrhizin (GL), a major component of licorice (*Glycyrrhiza glabra* L.), is ingested orally as a sweetener or as an ingredient in oriental medicine. GL has a steroid-like action and anti-viral activity (Pompei et al., 1979). Indeed, use of GL to treat chronic viral hepatitis has shown that it can reduce hepatocellular injury by immunopathologic mechanisms and hepatotoxins, possibly through its ability to inhibit phospholipase A2 activity preventing liver lysosome labilization²⁹⁻³⁰). GL is metabolized to GA, and then 3-epi-18-GA via 3-keto-18-GA by human intestinal flora³¹). GA shows pronounced anti-inflammatory activity and anti-estrogenic action via alteration of cortisol metabolism³²⁻³⁵). GA inhibits the

growth and stimulates melanogenesis in cultured melanoma cells³⁶). There are many reports about the use of differentiation factors for the resolution of uncontrolled growth such as in tumors, but there are few report about its use in the dental field including gingival hyperplasia.

The purpose of this study was to investigate the effects of GA on human gingival fibroblasts by monitoring cell growth and proliferation, total collagen synthesis, and nuclear acridine orange binding in human gingival fibroblasts.

II. Materials and Methods

1. Culture of human gingival fibroblasts

Human gingival fibroblasts were isolated with explant culture technique from patients undergoing orthodontic treatment by previously described methods and characterized by alkaline phosphatase activity³⁷⁻³⁸). Briefly, these gingival tissues were cut into 1mm³ explants and placed on a 100mm culture dishes (Falcon, Division of Becton Dickinson and Co., Lincoln Park, NJ, U.S.A.) containing 10,000 U/ml of penicillin G sodium, 10,000 µg/ml of streptomycin sulphate, 25 µg/ml of amphotericin B, and 10% heat-inactivated fetal bovine serum(FBS) at 37 °C in humidified atmosphere of 5% CO₂ and 95% air. After 2 or 3 days, fibroblasts started to outgrow from the explants. When the primary cell culture reached confluence, cells were detached with 0.025% trypsin and 0.05% EDTA, diluted with culture medium, and then subcultured in a ratio of 1:4. Cell cultures

between the 5th and 6th passage were used in this study. The viability of cells was verified utilizing the trypan blue exclusion method³⁹). Over 95% of viable cells were used in this study.

2. Cell morphology

Cells were plated in 35 mm dishes(Falcon) at 2×10^4 cells per dish and allowed to attach for 48 h. GA (Sigma Chemical Co., St. Louis, MO, U.S.A) was first dissolved in small volume of ethanol and diluted with culture medium to 0, 50 µg/ml and 100 µg/ml. This diluted GA was added to each culture plate and the cells were incubated for 24 or 48 h. The cell morphology was observed under an inverted-microscope(Olympus Optical Co., Tokyo, Japan) at the end of each experiment.

3. MTT assay

The cell growth and proliferation assay is dependent on the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically⁴⁰). The fibroblast cells were plated in 96-well dishes(Falcon) at 1×10^4 cells per well. Monolayer cultures in microtitration plates were incubated at 0, 50 and 100 µg/ml concentrations of GA. After 24 or 48 h incubation of fibroblast cells, 100µg of MTT was added to each well and then the plates were incubated at 37 °C in the dark for 4 h prior to

use. The supernatant of each well was vacuum - aspirated, and 1 ml of Dimethyl sulfoxide(DMSO) was added to each well. The plates were agitated to enhance the dissolution of the MTT - formazan. The mitochondrial dehydrogenase activity at each GA concentration was calculated as the percentage of the control activity from the absorbance values. The absorbance was measured at 570 nm in a microplate reader(Molecular Devices Co., Menlo Park, CA, USA). 0.1 M of phosphoric acid was used as a positive control.

4. Quantitative measurement of 4-hydroxyproline

To measure the collagen synthesis, 4 - hydroxyproline in the cell and medium was measured. The human gingival fibroblasts were homogenated in 6 N - HCl for the hydrolysis of cells and heated at 110 °C for 12 - 18 h⁴¹). After hydrolysis, the sample was then filtered through a No. 4 filter paper(Whatman Co., Maidstone, U.K.) on a funnel, and 50 $\mu\ell$ of filtrated fluid was transferred to a 5 ml glass vial. One ml of 60% isopropylalcohol and 84 mg chloramine T (Sigma) in 0.2 N acetate citric buffer were added to the vial and then incubated for 10 min at room temperature. After addition of p - Dimethylaminoben - zalde - hyde as chromogen, the vial was further incubated at 50 °C for 90 min, and the absorbance of supernatant measured at 558 nm. 0.1 M of phosphoric acid was used as a positive control.

5. Acridine orange binding assay

After treatment with GA for 24 h in vitro, the cells were centrifuged, resuspended in - minimal essential medium(- MEM), and maintained at 4 °C. Just before the flow cytometric analysis, cells were stained for 2 - 3 min with 0.1 mM acridine orange (Sigma) in - MEM. This procedure permits cellular staining of DNA without affecting cell viability^{42 - 43}). Acridine orange binding to DNA(>50,000 nucleated cells) was analyzed using a flow cytometer (Becton - Dickinson, Mountain View, CA, U.S.A.).

6. Flow cytometry

Acridine orange binding of DNA was measured using the flow cytometer instrument with an excitation wavelength of 488 nm. Green fluorescence emitted was measured at 515 - 560 nm. DNA contents per nucleus were measured by using the fluorescence of individual cells. A flow cytometric parameter was adjusted such that the mean green fluorescence of diploid cells was arbitrarily assigned a value between channels 90 - 100. The quantity of viable cells was measured using the fluorescence of individual cells. The cytometric parameter was adjusted such that the mean green fluorescence of viable cells was arbitrarily assigned a value between channels 180 - 200.

7. Statistics

Using the ANOVA and student's t - test, the statistical significance of the differences

in the proportion of cell growth, cell proliferation and collagen production.

III. Results and Discussion

Most of the control human gingival fibroblasts in culture exhibited normal stretched cytoplasmic processes and only a few cells were round. Treatment with 100 $\mu\text{g/ml}$ of GA for 24 h made human gingival fibroblasts round. Some cells detached from monolayer and floated into the growth medium. GA treatment might be related to the disappearance of cytoplasmic processes and the change into round shape.

The inhibitory effects of GA on human gingival fibroblasts were significantly higher than those of the control, and cell growth and proliferation was significantly inhibited at 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ of GA (Fig. 1).

The inhibition of cell growth and proliferation might result in the morphological change in gingival fibroblasts. This result supports the hypothesis that GA changes the morphotype of human gingival fibroblasts and inhibits the cell growth and proliferation in gingival fibroblasts by blocking selectin (a family of adhesion receptors) modulating signal transduction, and inhibiting arachidonic acid metabolism⁴⁴⁻⁴⁶).

GA inhibits the cell activity of human gingival fibroblasts in a concentration-dependent manner. The total collagen in the extracellular medium was increased and that in the cell was decreased in the presence of GA (Fig. 2). Hence, GA enhanced the release of collagen from cells into the extracellular medium. The released extracellular collagen might bind the membrane

receptors for collagen⁴⁷). After binding, the extracellular collagen may be related to the change of cell morphology, and regulate cell activity such as cell growth and proliferation, and total collagen synthesis in human gingival fibroblasts.

To investigate the molecular mechanism for change of cell morphology and the inhibition of cell activity, we carried out acridine orange binding assay by flow cytometry. This type of analysis is based on both morphometric and biochemical criteria⁴²) and permits cellular staining of DNA without affecting cell viability. Furthermore, this technique permits the analysis of treatment effects on a subpopulation of cells as opposed to biochemical procedures that quantitate an average effect for an entire cell population (see in materials and methods).

In the study of acridine orange binding by flow cytometry in human gingival fibroblasts at 24 h after GA treatment, diploid cells with a mean fluorescence over channel 80 decreased and cells with reduced acridine orange binding with a fluorescence below channel 80 increased (Fig. 3A and Fig. 3B). The decrease in cell viability and the acridine orange binding suggest that DNA degradation might occur in human gingival fibroblasts treated with GA⁴²). These results indicate that GA might be related to cytotoxic effect of fibroblast via DNA degradation⁴⁸⁻⁴⁹). Further study is needed to monitor the expression of HLA class I antigens in human gingival fibroblasts associated with cytotoxic effector function. Our previous study also showed that GA decreased the - blocker induced

calcium ion influx in human gingival cells⁵⁰). These effects of GA might be useful to modulate the drug - induced gingival change.

All these results suggest that GA may inhibit the cell activity of human gingival fibroblasts including collagen synthesis, and might be useful for the resolution of uncontrolled growth in dental field such as gingival hyperplasia at the concentration of about 50 $\mu\text{g}/\text{ml}$.

IV. Conclusion

We examined the effect of glycyrrhetic acid (GA), a cell differentiation factor for the resolution of uncontrolled growth, on the viability of cultured human gingival fibroblasts. Human gingival fibroblasts were harvested and isolated from patients undergoing orthodontic treatment. Cell growth and proliferation, collagen synthesis, and acridine orange binding of the nucleus of cultured fibroblasts in the presence of GA were examined by 3 - (4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) assay, the amount of 4 - hydroxyproline, and acridine orange binding by flow cytometry, respectively.

Morphologically, the fibroblasts treated with 100 $\mu\text{g}/\text{ml}$ of GA became round in shape. GA inhibited the growth and proliferation of human gingival fibroblasts at concentrations over 50 $\mu\text{g}/\text{ml}$. The total collagen inside the cell decreased in the presence of GA, and that in the extracellular medium increased in the presence of GA. When human gingival fibroblasts were cultured with 100 $\mu\text{g}/\text{ml}$ of GA for 24 h, diploid

cells with a mean fluorescence over channel 80 decreased and cells with reduced acridine orange binding (fluorescence below channel 80) increased.

These results suggest that GA can inhibit cell growth, proliferation and collagen synthesis, DNA degradation may be induced by GA in human gingival fibroblasts.

V. Acknowledgement

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VI. Referenxes

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Glycyrrhetic acid

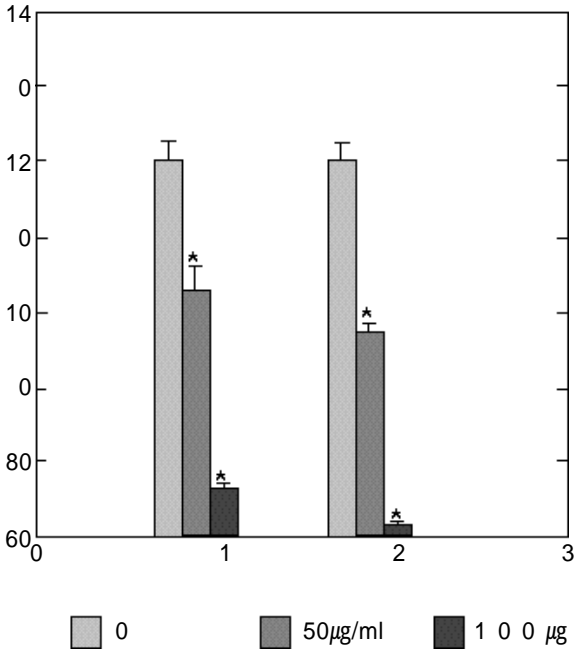


Fig. 1

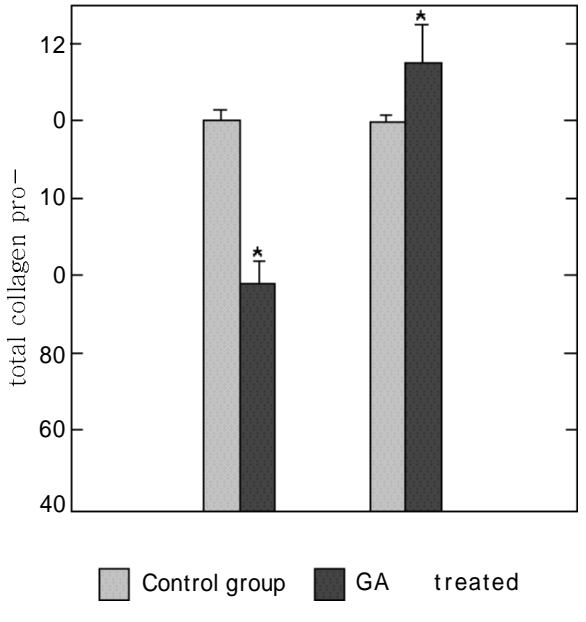


Fig. 2

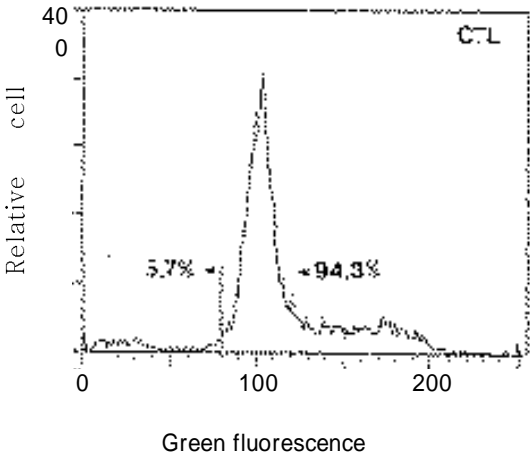


Fig. 3 (A)

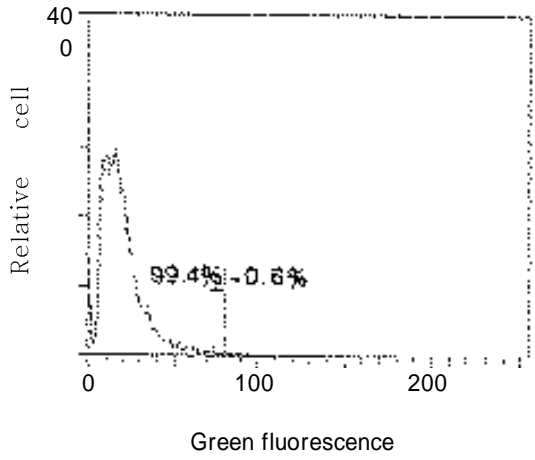


Fig. 3 (B)

- hyperplasia model and drug to treat gingival hyperplasia. *J. Oral Biol.* 18: 121 - 126, 1994.
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Fig. 1. Proportional change of cell growth and proliferation by GA in human gingival fibroblasts (%). GA - induced decrease of cell growth and proliferation was shown in human gingival cells. Cells (1×10^4 cells) were treated with 0, 50 and 100 $\mu\text{g}/\text{ml}$ concentrations of GA for 24 or 48 h in - MEM and then analysed by ELISA reader using 100 μg of MTT. The proportion of cell growth and proliferation was calculated as a percentage of the control activity from the absorbance values at 570 nm [Cell activity (%) = (Optical density of experimental group / Optical density of control group) \times 100]. Each column and vertical bar represent the mean + 1 SD of 12 samples. Significant differences from control, * $P < 0.01$.

Fig. 2. Proportional change of total collagen production by GA in human gingival fibroblasts (%). GA - induced release of total collagen from human gingival fibroblasts into medium was shown. Cells (1×10^4 cells) were treated with 100 $\mu\text{g}/\text{ml}$ concentrations of GA for 24 h in - MEM and then analysed by ELISA reader using 0.4% of SRB. The proportion of total collagen synthesis was calculated as a percentage of the control collagen synthesis from the absorbance values at 558 nm [Relative proportion of total collagen synthesis (%) = (Optical density of experimen -

tal group / Optical density of control group) \times 100]. Each column and vertical bar represent the mean + 1 SD of 3 samples. Significant difference between medium and cell, * $P < 0.01$.

Fig. 3. DNA histogram showing GA - induced loss of acridine orange binding in human gingival fibroblasts. Cells ($2 \times 10^6/\text{ml}$) were treated with 100 $\mu\text{g}/\text{ml}$ of GA for 24 h in - MEM and then analysed by flow cytometry using acridine orange staining method. Relative cell number was indicated on the vertical axis, and green fluorescence was indicated on the horizontal axis. Diploid cells had a mean fluorescence at channel 100 and cells with reduced acridine orange binding had a fluorescence below channel 80.

1, 2, 3, 1, 1,
4, 5, Hidomi kurihara⁶

1, 2,
3,
4,
5, Hirosima
6

,
acridine orange

acridine orange 3 - (4,5 -
dimethylthiazol - 2 - yl) - 2,5 - diphenyl
tetrazolium bromide (MTT) , 4 - hydrox -
yproline, acridine
orange

100µg/ml

50µg/ml

가

100µg/ml

24

, 80

diploid

가

80

acridine orange

가