Effects of $18\alpha$- and $18\beta$-glycyrrhetinic acid on apoptosis and proliferation by phenytoin and cyclosporin A in human gingival fibroblasts

REIRI TAKEUCHI¹, HIROKO MATSUMOTO¹, YOSHIKI AKIMOTO² and AKIRA FUJII³

Abstract: In this study, we investigated effects of $18\alpha$- and $18\beta$-glycyrrhetinic acid ($18\alpha$- and $18\beta$-GA), that induce apoptosis and inhibit proliferation in multiple types of cells, on apoptosis and proliferation of human gingival fibroblasts to establish the medication of phenytoin (PHT)- and cyclosporin A (CsA)-induced gingival overgrowth. It has been reported that the cause of this disease was related to depressed apoptosis and enhanced proliferation in gingival fibroblasts exposed to drugs. Cells were stimulated in serum-free DMEM for 1 week without stimulant, with only PHT (20 $\mu$g/mL) or CsA (10 $\mu$g/mL), and with PHT or CsA + $18\alpha$- or $18\beta$-GA (both 10 $\mu$M), and then cell apoptosis, viability, and proliferation were measured. $18\alpha$-GA induced apoptosis and cell-growth inhibition in gingival fibroblasts cultured with PHT and $18\beta$-GA also induced those in gingival fibroblasts cultured with CsA. Therefore, $18\alpha$-GA and $18\beta$-GA may apply for the medication of PHT- and CsA-induced gingival overgrowth respectively.

Key words: drug-induced gingival overgrowth, gingival fibroblast, apoptosis, cell proliferation

Introduction

Gingival overgrowth is one of the oral diseases that cause problems on both oral function and facial appearance. This disease, in response to antiepileptics (phenytoin, PHT), immunosuppressants (cyclosporin A, CsA), and calcium channel blockers (amlodipine, nifedipine, etc.), has characteristics of an increased number of fibroblasts and an accumulation of collagenous components in gingival connective tissue and epithelial hyperplasia with elongated, branched rete pegs penetrating into the connective tissue. In the previous studies, it has been reported that the cause of this disease was related to depressed apoptosis and enhanced growth in gingival fibroblasts exposed to these drugs, and inflammation in gingival tissue.

$18\alpha$- and $18\beta$-glycyrrhetinic acid ($18\alpha$- and $18\beta$-GA) are components of glycyrrhizin, a triterpene glycoside extracted from licorice root. $18\alpha$-GA and $18\beta$-GA have a variety of interesting activities such as an inhibitory effect on tumorous cell proliferation and an anti-inflammatory effect. It also has been reported that $18\alpha$-GA might apply for medicine of nifedipine-induced gingival overgrowth since it induced apoptosis and inhibited proliferation in fibroblasts isolated from overgrown gingival tissue.

Thus, we investigated effects of $18\alpha$-GA and $18\beta$-GA on apoptosis and proliferation of human gingival fibroblasts in the presence of PHT and CsA.

Materials and Methods

Materials

$18\alpha$-GA, $18\beta$-GA, PHT, and CsA were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Dulbecco’s
modified Eagle medium (DMEM), streptomycin, penicillin G, amphotericin B, trypsin-EDTA-4Na in Hanks solution, and fetal bovine serum (FBS) were purchased from Invitrogen Japan K.K. (Tokyo, Japan) and Sigma-Aldrich Japan K.K. APOPercentage™ Apoptosis Assay Kit and Vi-CELL® Reagent Pak were purchased from Biocolor Ltd. (Northern Ireland, UK) and Beckman Coulter K.K. (Tokyo, Japan), respectively.

**Cell culture**

Cultures of fibroblast-like cells were established from gingival specimens using methods described previously\(^{12}\). The specimens were obtained during extraction of the remaining teeth from a healthy volunteer. The protocol was approved by the Committee on Studies Involving Human Being of the Nihon University School of Dentistry at Matsudo (EC099-001). The patient gave written informed consent for use of their specimens. Cells were incubated in an atmosphere of 5% CO\(_2\) - 95% air at 37°C in culture medium, DMEM-10 (DMEM supplemented with 10% FBS, streptomycin 100 \(\mu\)g/mL, penicillin G 100 U/mL, amphotericin B 0.2 \(\mu\)g/mL), and routinely passaged with 0.25% trypsin and 1 mM EDTA-4Na in Hanks solution. The primary culture cells of the 8th to 10th passages were used for apoptosis and cell viability and proliferation assays. These assays were performed after that semi-confluent cells were incubated in serum-free DMEM for 1 week without stimulant (control), with only PHT (20 \(\mu\)g/mL) or CsA (10 \(\mu\)g/mL), and with PHT or CsA + 18\(\alpha\)-GA (10 \(\mu\)M) or 18\(\beta\)-GA (10 \(\mu\)M). It was referred to the following studies to determine the experimental conditions: Ref. 1 for PHT; Ref. 4 for CsA; Ref. 8 and 11 for 18\(\alpha\)- and 18\(\beta\)-GA.

**Apoptosis assay**

The apoptosis assay was carried out by APOPercentage™ apoptosis assay kit according to its manual, to monitor the occurrence of cell apoptosis in the presence of PHT or CsA with/without 18\(\alpha\)-GA and 18\(\beta\)-GA. After cells were treated with PHT, CsA, 18\(\alpha\)-GA, and 18\(\beta\)-GA, medium was removed, and then fresh DMEM supplemented with APOPercentage Dye was added to plates. Following one-hour incubation with the dye, medium was removed, APOPercentage Dye release reagent was added and then plates were gently shaken for 10 min. The absorbance of cell-bound dye recovered into solution was then measured using a micro plate reader at 550 nm.

**Cell viability assay**

Cell viability assay was carried out by Vi-CELL® reagent pak and Vi-CELL™ XR cell viability analyzer in the presence of PHT or CsA with/without 18\(\alpha\)-GA and 18\(\beta\)-GA. After cells were treated with/without PHT, CsA, 18\(\alpha\)-GA, and 18\(\beta\)-GA, the percentage of viable cells was determined with trypan blue dye.

**Cell proliferation assay**

Cell proliferation assay was carried out by Vi-CELL® reagent pak and Vi-CELL™ XR cell viability analyzer in the presence of PHT or CsA with/without 18\(\alpha\)-GA and 18\(\beta\)-GA. After cells were treated with/without PHT, CsA, 18\(\alpha\)-GA, and 18\(\beta\)-GA, the total number of cells was counted.

**Statistical analysis of data**

All data were expressed as the mean ± S.D. Statistical significance was determined by Student’s t-test with Bonferroni’s correction for multiple comparisons. \(P < 0.05\) was considered to be significant.

**Results**

Effects of 18\(\alpha\)- and 18\(\beta\)-glycyrrhetin acid on apoptosis and viability and proliferation of human gingival fibroblasts in the presence of phenytoin

Cells were treated in serum-free DMEM with/without PHT ± 18\(\alpha\)- or 18\(\beta\)-GA for 1 week. As shown in Figs. 2A, 2B, and 2C, the apoptosis was significantly inhibited and the viability and proliferation were sig-
Effects of 18α- and 18β-GA on gingival overgrowth by PHT or CsA

Significantly increased in gingival fibroblasts exposed to PHT compared with the untreated control cells. Both 18α-GA and 18β-GA significantly elevated PHT-depressed apoptosis. Only 18α-GA significantly decreased PHT-enhanced cell viability and proliferation. 18β-GA showed no effect on PHT-increased cell viability and proliferation.

Effects of 18α- and 18β-glycyrrhetinic acid on apoptosis and viability and proliferation of human gingival fibroblasts in the presence of cyclosporin A

Cells were treated in serum-free DMEM with/without CsA ± 18α- or 18β-GA for 1 week. As shown in Figs. 3A, 3B, and 3C, the apoptosis was lower and the viability and proliferation were higher in the cells treated with CsA than the untreated control cells significantly. Only 18β-GA significantly induced apoptosis and increased viability in the cells treated with CsA. Both 18α-GA and 18β-GA significantly enhanced the proliferation in CsA-treated cells. 18α-GA showed no effect on CsA-inhibited apoptosis and increased cell viability.

Discussion

In this study, we examined whether 18α-GA and 18β-GA have an effect on apoptosis and proliferation of human gingival fibroblasts to establish the medication of PHT- and CsA-induced gingival overgrowth. 18α- and 18β-GA induce apoptosis and cell-growth inhibition in multiple types of cancer cells, including human stomach cancer cells, promyelocytic leukemia HL-60 cells, hepatoma cells, and cervix and uterus tumor cell line SiHa cell [13]. GA also can inhibit tumor cell growth without hindering normal cell growth [14]. And 18α-GA induces apoptosis and inhibits growth in gingival fibroblasts derived from gingival overgrowth caused by nifedipine in a patient without influence on normal gingival fibroblasts [8, 11].

In the previous study, it has been demonstrated that serum starvation induced apoptosis and growth-inhibition in bovine skin fibroblasts [15]. We found the similar observation in human gingival fibroblasts cultured with serum-free medium for 1 week (data not shown). Figs. 2 and 3 show that PHT and CsA inhibit apoptosis and increase cell viability and proliferation in the cells

Fig. 2 Effects of 18α-GA and 18β-GA on apoptosis (A), viability (B), and proliferation (C) in human gingival fibroblasts treated with PHT. Semi-confluent cells were incubated in serum-free DMEM for 1 week without stimulant (control), with only 20 μg/mL of PHT, and with 20 μg/mL of PHT + 10 μM of 18α- or 18β-GA. And then apoptosis (n = 4) and cell viability (n = 5) and cell proliferation (n = 5) were assessed. Cell viability and proliferation are expressed by the percentage of viable cells in the total and by the ratio of number of cells in the stimulated group to in the control group respectively. Data are shown as mean ± S.D. *p < 0.05, **p < 0.01 rs. the untreated control group; *p < 0.05 rs. PHT-stimulated group.
under the same conditions. These results are similar to the previous investigation\(^{6,16,17}\). Results also show the inhibitory effects of 18α-GA on PHT-depressed apoptosis and enhanced proliferation in gingival fibroblasts, and the inhibitory effects of 18β-GA on those by CsA.

It has been reported that the mechanism of drug-induced gingival overgrowth was the variations in the balance between apoptosis and proliferation in gingival fibroblast cells. In vivo study, it has been shown the diminished apoptosis and increased proliferation with decrease in caspase-3 and FOXO1 expressions in all specimens of PHT-, CsA-, nifedipine-induced gingival overgrowth\(^5\). Sano et al. have reported that PHT and nifedipine induced proliferation of cultured gingival fibroblasts from human with gingival overgrowth to these drugs by endogenously generated endothelin (ET)-1 possibly via ETA receptors\(^{18}\). In vitro, it has also been reported that CsA-induced gingival overgrowth was caused by the decreased apoptosis through inhibition of cytochrome c, caspases-3 and -9, Bax, and Fas-L with upregulated Bcl-2 in human gingival fibroblasts more than the increased proliferation\(^4\). Parkar et al. have demonstrated that CsA enhanced cell-cycle progression of gingival fibroblasts by up-regulation of cyclin B1 in vitro\(^{19}\). 18α- and 18β-GA may inhibit the above-mentioned mechanism by PHT and CsA. Also regarding the medication, it has been suggested that azithromycin might improve CsA-induced gingival overgrowth by blocking human gingival fibroblasts proliferation and collagen synthesis\(^{20}\).

Therefore, 18α-GA and 18β-GA might apply for the medication of gingival overgrowth induced by PHT and CsA respectively.

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**References**


我々はこの研究で、フェニトイン（PHT）およびシクロスポリン A（CsA）による歯肉増殖症の薬物療法を目指して、ヒト歯肉線維芽細胞を用い、これら薬物のアポトーシス抑制・細胞増殖促進作用に対する$18\alpha$および$18\beta$-グリチルレチン酸（$18\alpha$および$18\beta$-GA）の効果を検討した。細胞はPHT（20μg/mL）、CsA（10μg/mL）、$18\alpha$または$18\beta$-GA（10μM）添加の無血清培地で一週間培養され、その後、アポトーシス細胞数、生細胞数、増殖能が測定された。$18\alpha$-GA はPHT 存在下で培養された細胞のアポトーシスを誘導し増殖を抑制した。そして$18\beta$-GA はCsA 存在下の培養細胞に対して同様の効果を示した。これらの結果から、$18\alpha$-GA はPHT による、$18\beta$-GA はCsA による歯肉増殖症の治療薬として応用が可能であろうと考えられた。

キーワード：薬物性歯肉増殖症、歯肉線維芽細胞、アポトーシス、細胞増殖