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Retinoic Acid Increases the Cell Cycle Progression of Human Gingival Fibroblasts by Increasing Cyclin E and CDK 2 Expression and Decreasing $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ Expression

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Retinoic acid plays an important role in the regulation of cell growth and differentiation. In our present study, we evaluated the effects of all-*trans* retinoic acid (RA) on cell proliferation and on the cell cycle regulation of human gingival fibroblasts (HGFs). Cell proliferation was assessed using the MTT assay. Cell cycle analysis was performed by flow cytometry, and cell cycle regulatory proteins were determined by western blot. Cell proliferation was increased in the presence of a 0.1 nM to 1 μM RA dose range, and maximal growth stimulation was observed in cells exposed to 1 nM of RA. Exposure of HGFs to 1 nM of RA resulted in an augmented cell cycle progression. To elucidate the molecular mechanisms underlying cell cycle regulation by RA, we measured the intracellular levels of major cell cycle regulatory proteins. The levels of cyclin E and cyclin-dependent kinase (CDK) 2 were found to be increased in HGFs following 1 nM of RA treatment. However, the levels of cyclin D, CDK 4, and CDK 6 were unchanged under these conditions. Also after exposure to 1 nM of RA, the protein levels of $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ were decreased in HGFs compared with the control group, but the levels of p53 and pRb were similar between treated and untreated cells. These results suggest that RA increases cell proliferation and cell cycle progression in HGFs via increased cellular levels of cyclin E and CDK 2, and decreased cellular levels of $p21^{WAF1/CIP1}$ and $p16^{INK4A}$.

Keywords: Retinoic acid, Cell cycle, Human gingival fibroblasts

Introduction

Retinoic acid plays a fundamental role in embryogenesis, reproduction, vision, and control of cell growth and differentiation of many adult tissues [1-3]. Topical administration of retinoic acid improves several features of skin, including fine and deep wrinkles, texture, and color [4]. Histologic examination of retinoic acid treated skin reveals epidermal thickening with decreased keratinocyte differentiation, deposition of glycosaminoglycan-like materials in the epidermis, increased cellularity in the dermis, synthesis of new extracellular matrix, and fibroblast activation [5]. Previous studies have shown that retinoic acid induces collagen synthesis in human dermal fibroblasts, and that topical application of retinoic acid to human skin prevents

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induction of a number of metalloproteinase involved in destruction of the extracellular matrix [6]. In the course of screening for biological activities of retinoic acid, we found that all-*trans* retinoic acid (RA) has potent cell proliferation activity of human gingival fibroblasts (HGFs) via cell cycle regulation. The proliferation of HGFs is important for wound healing process of gingiva after injury.

The cell proliferation results from cell cycle progression. Progression around the cell cycle is governed by a family of cyclin-dependent kinases (CDKs) and the cyclins [7,8]. As cells enter the cell cycle from G0/G1, cyclin D and E are synthesized sequentially and both are rate-limiting for S phase entry. A key role of CDKs is to inactivate, by phosphorylation, negative regulators of progression, notably pRb, to permit exit from G1 and entry into S phase. Cyclin D bind pRb directly, with pRb being the critical substrate of CDK 4 and CDK 6, although cyclin E/CDK 2 also phosphorylate pRb. Phosphorylation of pRb relieves its inhibitory effect on the transactivation function of E2F family transcription factors that are required for S phase. CDK activity is counterbalanced by a variety of low molecular weight cyclin-dependent kinase inhibitors (CKIs), p53, $p21^{WAF1/CIP1}$, and $p16^{INK4A}$. $p16^{INK4A}$ is a specific inhibitor of CDK 4 and 6, and regulates cell cycle progression negatively [7,9]. $p21^{WAF1/CIP1}$ binds and inhibits a broad range of active cyclin-CDK complexes including cyclin D-CDK 4, cyclin D-CDK 6, and cyclin E-CDK 2 [7]. $p21^{WAF1/CIP1}$ is a critical down effector in the p53-specific pathway of growth suppression in mammalian cells. Transcription of the $p21^{WAF1/CIP1}$ gene is directly activated by p53, and its protein product can bind to CDKs and inhibits their actions. However, $p21^{WAF1/CIP1}$ can be also regulated via p53-independent pathway [10].

In the present study, we show that the RA increases the cell cycle progression of HGFs. In addition, to investigate the mechanism of RA-induced cell cycle progression, we examined the intracellular levels of cell cycle regulatory proteins such as cyclin D, CDK 4, CDK 6, cyclin E, CDK 2, p53, $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and pRb.

Materials and methods

Culture of HGFs

HGFs were isolated with an explant culture technique from patients undergoing orthodontic treatment by previously described methods [11]. Briefly, these gingival tissues were cut into 1 mm^2 explants and placed on a 100 mm culture dishes (Falcon, Division of Becton Dickinson and Co., Lincoln Park, NJ, USA) 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; Gibco BRL, Grand Island, NY, USA) at 37℃ in a humidified atmosphere of 5% CO_2 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. All-*trans* retinoic acid (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in DMSO (Sigma Chemical Co.) before dilution in culture media. The final concentration of DMSO in culture media was 0.01%. Control cells were treated with media containing 0.01% DMSO.

Cell proliferation assay

Cell proliferation by RA was determined by viable cell counting [12]. Cultured HGFs were plated at 2×10^4 cells per well of a 6-well culture plate. After 24 h, cells were exposed to 0.1 nM - 10 mM of RA. The number of viable cells after trypan blue exclusion was counted after 2 and 4 days of incubation at 37°C. There were four cultures in each group at each time.

Cell cycle analysis

Approximately 70% confluent monolayer cells in 100-mm Petri dishes were harvested by trypsinization and washed with phosphate buffered saline (PBS). Cells were then fixed in cold 70% ethanol for 45 min at 4°C, pelleted, resuspended in TSP solution (0.1% Triton X-100, 0.1% sodium citrate and 0.005% propidium iodide) containing 1 μg/ml RNase A and incubated for 30 min at room temperature. The state of the cell cycle was then analyzed on FACScan flow cytometer (Beckton-Dickinson and Co., San Jose, CA, USA).

Western blot analysis

Cells grown in 100-mm Petri dishes were lysed in a lysis buffer [10 mM Na_2HPO_4 (pH 7.2), 0.9% NaCl, 1%

Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide and 0.004% sodium fluoride] on ice for 15 min. The cell lysate was centrifuged at 15,000 × g for 20 min and the supernatant containing 1 μg/ml of protein was denatured by boiling for 3 min in a sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.001% bromophenol blue]. An aliquot of the denatured supernatant containing 50 mg of protein was electrophoresed in a 15% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore Corp., Bedford, MA, USA). After incubation in ablocking solution (Zymed, San Francisco, CA, USA) for 1 h at room temperature, the membrane was exposed to the following antibodies (diluted 1/1000): a rabbit anti-cyclin D1 monoclonal antibody (Santa Cruz Biotechnology Santa Cruz, CA, USA), a rabbit anti-CDK 4 polyclonal antibody (Santa Cruz Biotechnology), a rabbit anti-CDK 6 polyclonal antibody (Santa Cruz Biotechnology), a mouse anti-cyclin E polyclonal antibody (Oncogene Science, Uniondale, NY, USA), a rabbit anti-CDK 2 polyclonal antibody (Santa Cruz Biotechnology), a mouse anti-p53 monoclonal antibody (Ab-2; Oncogene Science), a mouse anti-$p21^{WAF1/CIP1}$ monoclonal antibody (Santa Cruz Biotechnology), a mouse anti-pRb(IF8) monoclonal antibody (Santa Cruz Biotechnology), and a rabbit anti-$p16^{INK4A}$ polyclonal antibody (Santa Cruz Biotechnology) at room temperature for 90 min. After washing with the phosphate buffered saline (PBS), the membrane was treated with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology) for 1 h and again washed with PBS. The blot was developed by using the enhanced chemiluminescence (ECL) kit (Amersham Corp., Arlington Heights, IL, USA).

Statistical analysis

Data were analyzed using the statistical package for social sciences (SPSS). Values were expressed as the mean ±S.E.M. Statistical analyses were performed with Student's t test, and differences were considered significant at $P < 0.05$.

Results

RA enhances proliferation of HGFs

To investigate the effect of RA on cell growth of HGFs, rapidly proliferating HGFs were exposed to 0.1 nM - 1 mM of RA. Cell proliferation was increased in the presence of 0.1 nM - 1 mM of RA compared to the control (Fig. 1). Interestingly, maximal growth stimulation was observed in these cells exposed to 1 nM of RA. However, augmentation of cell proliferation was inhibited higher than 100 nM of RA compared to that in the group treated with 1 nM of RA.

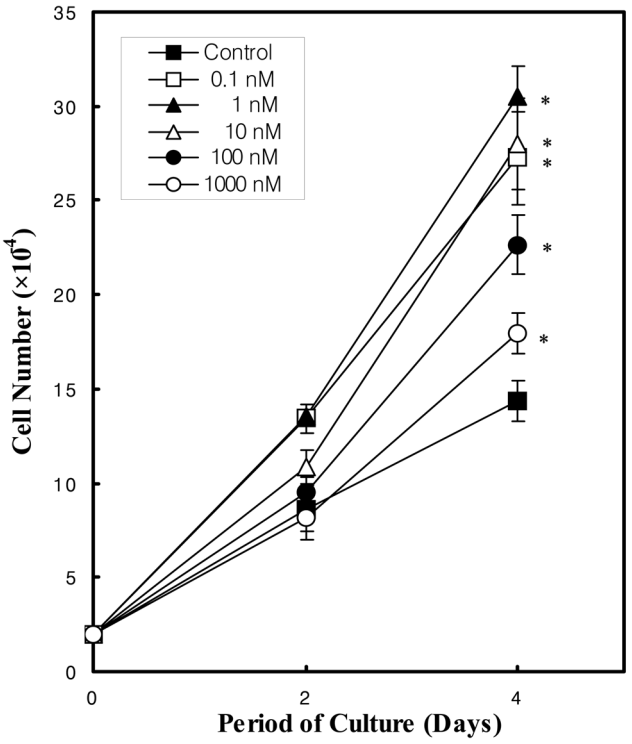

Fig. 1. Effect of RA on proliferation of HGFs. Cell numbers were counted by hemocytometer after 2 and 4 days incubation. Values are mean ±S.E.M. of four independent experiments. * $P < 0.05$ compared to control group.

RA increases the cell cycle progression

Since 1 nM of RA increase the cell proliferation, we examined the effect of 1 nM of RA on cell cycle. Cultured HGFs were exposed to 1 nM of RA for 2 days and processed for FACScan analysis as described in "Material and methods". RA altered the cell cycle distribution of growing HGFs. When the cells were cultured in the medium containing 1 nM of RA, the percentage of cells in S phase was increased approximately 2- to 3- fold compared to control group (Fig. 2). However, the percentage of cells in G0/G1 phase was decreased after 1 nM of RA treatment (Fig. 2). These results suggest that 1 nM of RA may increase the cell cycle progression of HGFs.

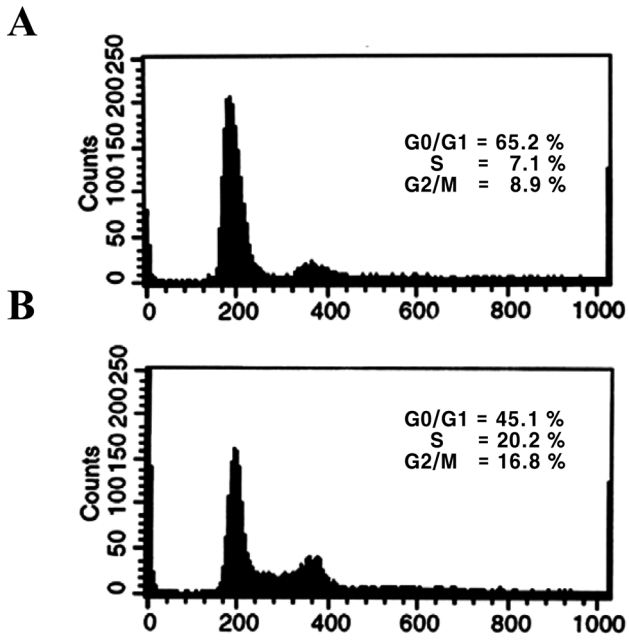

Fig. 2. Effect of RA on cell cycle progression of HGFs. RA induced cell cycle progression of HGFs. The cell cycle analysis was performed in HGFs after treatment with (B) or without (A) 1 nM of RA.

RA increases the intracellular levels of cyclin E and CDK 2, but does not alter the levels of cyclin D, CDK 4, and CDK 6

Since 1 nM of RA increased the percentage of cells in S phase and decreased the percentage of cells in G0/G1 phase, we investigated whether RA selectively regulates the intracellular levels of cell cycle regulators. The cyclin E level of HGFs exposed to 1 nM of RA was upregulated, and the CDK 2 level was also increased after 1 nM of RA treatment (Fig. 3-A). The protein levels of cellular cyclin D, CDK 4, and CDK 6 in RA-treated HGFs were similar to those in the control group (Fig. 3-B).

RA decreases the intracellular levels of p53 and $p16^{INK4A}$, but does not alter the levels of $p21^{WAF1/CIP1}$ and pRb.

After exposure to 1 nM of RA, the protein level of p53 was similar to that of control group, but the level of $p21^{WAF1/CIP1}$ was decreased in the cell treated with 1 nM of RA (Fig. 4-A). The cellular level of $p16^{INK4A}$ was dec-

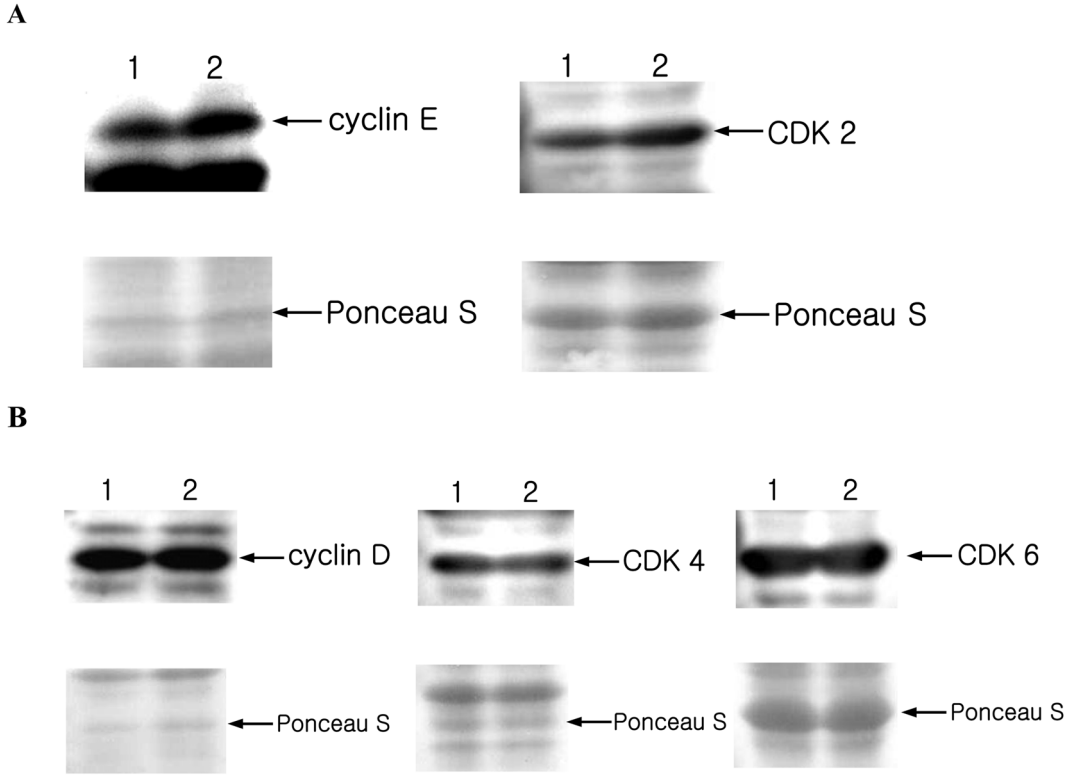

Fig. 3. Western blot analysis for intracellular levels of (A) cyclin E and CDK 2 , (B) cyclin D, CDK 4, and CDK 6 in cultured HGFs supplemented with RA for 2 days (1; control group, 2; experimental group). Cell extract equivalent to 100 ❍g of total cellular protein of HGFs was electrophoresed and transferred to a PVDF membrane. The intracellular protein levels of (A) cyclin E and CDK 2 , (B) cyclin D, CDK 4, and CDK 6 in HGFs were probed with respective antibodies diluted by 1 : 1000. After probing, the membrane was stained with 1×Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane (lower panel).

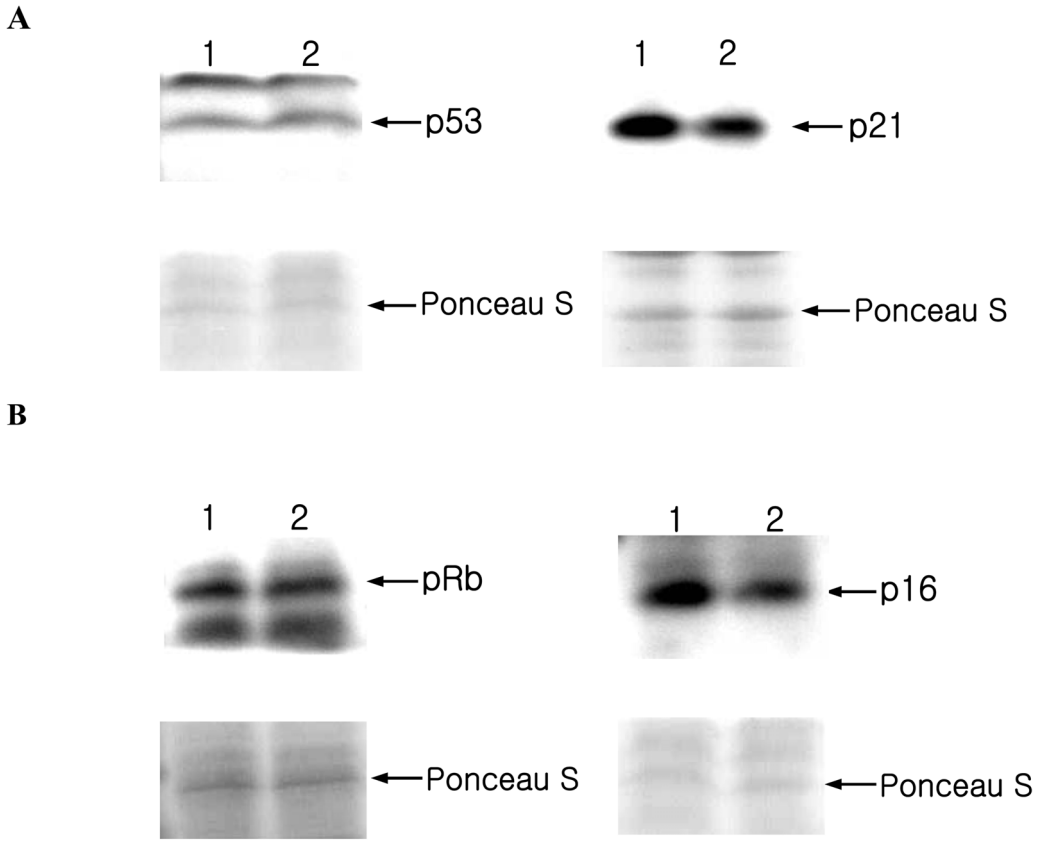

Fig. 4. Western blot analysis for intracellular levels of (A) p53 and p21 ,(B) pRb and p16 in cultured HGFs supplemented with RA for 2 days (1; control group, 2; experimental group). Cell extract equivalent to 100 ❍g of total cellular protein of HGFs was electrophoresed and transferred to a PVDF membrane. The intracellular protein levels of (A) p53 and p21 ,(B) pRb and p16 in HGFs were probed with respective antibodies diluted by 1 : 1000. After probing, the membrane was stained with 1×Ponceau S stain for 10 min to reveal the total cellular protein loaded per each lane (lower panel).

reased after exposure to 1 nM of RA, but the level of pRb protein was unchanged (Fig. 4-B).

Discussion

Retinoic acid has profound effects on the skin [13]. It induces regeneration of the dermal collagenous matrix and inhibits matrix metalloproteinase [6]. However, the effects of retinoic acid on cell cycle progression of HGFs and the mechanism of its action in HGFs have not been elucidated. In the present study, we showed that the effect of the RA on cell proliferation and cell cycle regulation in HGFs. Cell proliferation was increased in HGFs exposed to 0.1 nM − 1 mM of RA compared to the vehicle control, and maximal increase was observed at 1 nM of RA. Cell cycle analysis showed that 1 nM of RA increased the percentage of cells in S phase and decreased the percentage of cells in G0/G1 phase. The results of the present study provide the evidence that exposure of HGFs to 1 nM of RA may result in an increase of cell proliferation and cell cycle progression.

The molecular mechanisms of cell cycle progression are not fully understood, but recent reports showed that cell cycle is controlled by some cell cycle regulatory proteins [7,8]. To investigate the mechanisms by which RA increases the cell cycle progression of HGFs, the levels of cyclin D, CDK 4, CDK 6, cyclin E, CDK 2, p53, $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and pRb that may be involved in regulation of replicative senescence were examined. Previous report has indicated that cyclin E, a nuclear protein, regulates G1-S transition [7]. In cell cycle, expression of cyclin E rises to a maximum level in late G1 and it associates with and activates CDK 2. The cyclin E-CDK 2 complex shows strong kinase activity shortly before cells enter S phase and leads to further phosphorylation on the pRb protein.

In a similar manner, cyclin D interacts with CDK 4 or CDK 6 to generate the cyclin D-CDK 4 or 6 complex which directly phosphorylate the pRb. In the present study, the levels of cyclin E and CDK 2 were notably increased by 1 nM of RA, but the levels of cyclin D, CDK 4 and CDK 6 were not changed. These data indicate that cyclin E and CDK 2 may be, in part, responsible for the RA-induced cell cycle progression in HGFs. It is known that the cell proliferation is regulated in part by the protein product of the $p16^{INK4A}$ tumor suppressor gene [7]. $p16^{INK4A}$ is involved in cell cycle regulation through the retinoblastoma pathway, and acts at the G1-S interface. The $p16^{INK4A}$ protein binds to CDK 4 and CDK 6 inhibiting the catalytic activity of the cyclin D-CDK 4 or 6 complexes that normally mediate passage through the G1 phase of the cell cycle by phosphorylation of pRb. Thus, loss of $p16^{INK4A}$ protein causes loss of the capacity to inactivate the cyclin D-dependent kinase that results in the inability of the cell to activate pRb, that ultimately leads to a loss of control over cell cycle progression. In this experiment, cellular $p16^{INK4A}$ level was decreased in the presence of 1 nM of RA. This observation suggests that $p16^{INK4A}$ may be important in the control of cell cycle regulation by RA in HGFs. Immunoblot analyses demonstrated that 1 nM of RA decreased the protein level of $p21^{WAF1/CIP1}$ concomitantly with cell cycle progression in HGFs, but the levels of p53 were not changed. Previous reports have indicated that p53 can increase the expression of $p21^{WAF1/CIP1}$ [7]. However, our p53 and $p21^{WAF1/CIP1}$ results are different from those reports. These conflicting results may be due to differences in cell types. Also, recent report showed that $p21^{WAF1/CIP1}$ can be regulated via p53-independent pathway [10].

These results obtained in the present study provide some evidences that RA increases the cell proliferation and cell cycle progression in HGFs, which is linked to an increased cellular levels of cyclin E and CDK 2, and decreased the cellular levels of $p^{16INK4A}$ and p21. Further studies are necessary to clarify the possibility that RA may be active in the healing process of gingiva after injury.

Acknowlegements

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