Arecoline suppresses HaCaT cell proliferation through cell cycle regulatory molecules

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Abstract. Betel nut chewing is the most common cause of oral submucous fibrosis (OSF). Arecoline is the main component of the betel nut, and is associated with the occurrence and development of OSF through cytotoxicity, genotoxicity and DNA damage. Similar types of stimuli elicit differential responses in different cells. In the present study, we investigated the effects of arecoline on the HaCaT epithelial and Hel fibroblast cell lines. The data showed that arecoline affected HaCaT cell morphology. MTT assay revealed that arecoline suppressed HaCaT cell proliferation. Furthermore, we found that arecoline induced the cell cycle arrest of HaCaT cells. In comparison with the untreated control cells, following treatment with ≥75 μg/ml arecoline an increased percentage of HaCaT cells remained at the G_0/G_1 phase of the cell cycle, accompanied by a reduced percentage of cells in the S phase. However, arecoline treatment did not significantly alter Hel cell cycle distribution. In the HaCaT epithelial cells, arecoline downregulated expression of the G_1/S phase regulatory proteins cyclin D1, CDK4, CDK2, E2F1 as determined by reverse transcription-PCR analysis and western blotting. In summary, arecoline inhibits HaCaT epithelial cell proliferation and survival, in a dose-dependent manner, and cell cycle arrest in the G_1/S phase, while this is not obvious in the Hel fibroblast cells. Potentially, our findings may aid in the prevention of arecoline-associated human OSF.

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Abbreviations: OSF, oral submucous fibrosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; E2F1, E2F transcription factor 1; CDK4, cyclin-dependent kinase 4; CDK2, cyclin-dependent kinase 2

Key words: oral submucous fibrosis, proliferation, carcinogenesis, cell cycle

Introduction

Oral submucous fibrosis (OSF) is a chronic insidious disease predisposing to cancer. The rate of carcinoma incidence in OSF cases reaches up to 7.6%. Areca nut (betel) chewing is one of the primary etiologic factors for OSF and carcinogenesis. The areca nut is indentified as a first class carcinogen by IARC (1,2). Histologically, most OSF is characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria while a minority is characterized by epithelial atypical hyperplasia. In tissues with OSF-associated carcinogenesis, both epithelial atrophy and atypical epithelia can exist or coexist. There is abnormal expression of proteins related to cell cycle arrest and apoptosis in OSF and in tissues showing carcinogenesis. The role and mechanism of arecoline in epithelial atrophy and epithelial hyperplasia require further investigation (3-10).

Eukaryotic cell division is a precise and orderly process with multi-factorial controls. The cell cycle can be divided into the four phases known as G_1, S, G_2 and M, with two important restriction points. The restriction points are G_1/S and G_2/M phase, and only across these two limiting points can there be final completion of cell replication and division. Normal cells with DNA damage and cells affected by other external stimuli can undergo G_1/S and/or G_2/M phase blockage, delaying progression of the cell cycle. Yet, these normal cells win enough time to repair DNA damage before DNA replication and cell division (11). Cyclins and expression of cyclin-dependent-kinase (CDKs) form an active complex which positively regulates the cell cycle. CDK inhibitors (CKIs) combine with CDKs, and inhibit the activity of CDKs, thus playing a role as a negative regulator of the cell cycle. Cyclin D/CDK4/6 complexes are necessary to promote cell cycle through the G_1/S phase restriction point. Cyclin E and cyclin A further promote the phosphorylation of Rb, induction of E2F1 in the intracellular accumulation, and promote a series of cyclins and cyclin-dependent protein kinases in the cell across the late G_1 phase to S phase. The ultimate target of the G_2 checkpoint signaling pathway is the cyclin-dependent kinase (CDK) complex, CDK1-cyclin B1. Deregulation of cell cycle machinery is an important step in malignant transformation. Cyclin D1 is one of the most important proto-oncogenes

Table I. Sequences of the primers for RT-PCR.

Gene	Forward primer	Reverse primer	Product (bp)
CDK2	CCTGGAGATTCTGAGATTGA	GGGAAACTTGGCTTGTAAT	113
CDK4	GCATCCCAATGTTGTCCG	AGGCAGCCCAATCAGGTC	499
Cyclin A	CCTGCGTTCACCATTCAT	TCTTCTCCTACTTCAACTAACC	383
Cyclin B	TTGGTTGATACTGCCTCTC	TCTGACTGCTTGCTCTTC	204
Cyclin D1	GAACAGAAGTGCGAGGAG	GCGGTAGTAGGACAGGAA	477
Cyclin E	TGGATGTTGACTGCCTTG	TCTATGTCGCACCACTGA	115
E2F1	CACTGAATCTGACCACCAA	ACCATAACCATCTGCTCTG	400
CDC2	AGAGTTCTTCACAGAGACT	GGATGATTCAGTGCCATT	488
β-actin	CCGTGACCTGACTGACTACCTC	ATACCGCAAGATTCCATACCC	276

RT-PCR, reverse transcription-PCR; CDK2, cyclin-dependent kinase 2; CDK4, cyclin-dependent kinase 4.

and cell cycle regulators. Its protein is known as cyclin D1 and is expressed in the G_1 phase of the cell cycle (12,13).

Similar types of stimuli elicit different responses in different cells. In the present study, we investigated the effects of arecoline on the HaCaT epithelial and Hel fibroblast cell lines. First, human keratinocyte cells of the HaCaT cell line and human embryo lung fibroblasts of the Hel cell line were treated with high doses of arecoline for a short time to observe the survival rate, cell cycle distribution and apotosis. Secondly, the molecular mechanism of epithelium atrophy induced by arecoline was studied.

Materials and methods

Cell lines. Keratinocytes of the naturally immortalized normal cell line, HaCaT, were obtained from the Laboratory of Apoptosis, College of Life Science, Hunan Normal University, China. The human embryonic lung fibroblast cell line (human normal fibroblast cell line), Hel, was established by the Laboratory of Molecular Virology, College of Life Science, Hunan Normal University, China (14).

Cell proliferation assay. The impact of arecoline treatment on cell proliferation was measured by the MTT assay as described previously (15). Briefly, keratinocytes from the natural immortalized normal cells, HaCaT, and human embryonic lung fibroblast cells, Hel, (10^4 cells/well) were cultured in triplicate with 10% FCS RPMI-1640 in 96-well plates in the presence or absence of 25, 50, 75, 100 and 125 μg/ml arecoline for 0, 24, 48 and 72 h, respectively. The cells were then exposed to 5 mg/ml MTT for 4 h. The generated formazan was dissolved with dimethyl sulfoxide, and the absorbance was measured at 570 nm using an ELX-800 microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA).

Flow cytometric analysis of the cell cycle. The previously described keratinocytes and lung fibroblast cells were cultured in 10% FCS RPMI-1640 to ~70% confluence and then treated with, or without, 25, 50, 75, 100 and 125 μg/ml arecoline for 12 h, respectively. The adherent cells were trypsinized, harvested and fixed in 70% ethanol at 4˚C. Subsequently, the cells were washed with cold PBS and stained with propidium iodide (PI) in working solution (0.5 mg/ml RNase, and 0.1 mg/ml PI in PBS). The cell cycle was characterized by flow cytometric analysis using a FACScan cytofluorimeter, and the data were analyzed by the CellQuest Pro Software (Becton-Dickinson, San Jose, CA. USA).

Reverse transcription-PCR analysis. Both cell lines were cultured in RPMI-1640 to 80% confluence and then treated with, or without, 25, 50, 75, 100 and 125 μg/ml arecoline for 12 h, respectively. The cells were harvested, and their total RNA was extracted. One microgram total RNA was reversely transcripted into cDNA, as described previously (15,16) and was subsequently used as the template for PCR reaction. The first step in the PCR reaction involved denaturing at 94˚C for 5 min, followed by 30 cycles of 94˚C for 45 sec, 55˚C for 45 sec, and 72˚C for 1 min, followed by 72˚C for 7 min for CDK2, CDK4, cyclin A, cyclin B, cyclin D1, cyclin E, E2F1 and CDC2. The primers CDK2, CDK4, cyclin A, cyclin B, cyclin D1, cyclin E, E2F1, CDC2 and β-actin were synthesized as shown in Table I. All RT-PCR reactions were repeated at least three times with varying cycle numbers to avoid potentially false results. β-actin was used as an endogenous control for normalization. The PCR products were analyzed by agarose-gel electrophoresis and ethidium bromide staining.

Western blotting. The ketatinocytes and the epithelial cells were cultured in RPMI-1640 up to 80% confluence and then treated with, or without, 25, 50, 75, 100 and 125 μg/ml arecoline for 12 h, respectively. The cells were harvested and lysed in the lysis buffer [1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 400 mM NaCl, 10% glycerol plus complete protease inhibitor mixture (Roche Diagnostics)]. The protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA). The cell lysates (50 μg) from individual samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (HyClone Laboratories, Logan, UT, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5, and 0.05% Tween-20) and probed with the primary antibody overnight at 4˚C. After washing with Tris-buffered saline/Tween-20 three times, the membranes were incubated

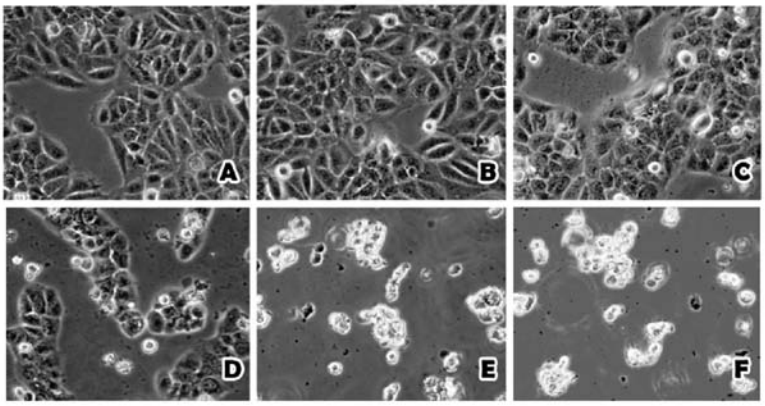

Figure 1. Arecoline affects the morphology of HaCaT epithelial cells, and promotes cell death in a dose-dependent manner. Concentrations of arecoline: (A) 0 μg/ml, (B) 25 μg/ml, (C) 50 μg/ml, (D) 75 μg/ml, (E) 100 μg/ml and (F) 125 μg/ml (magnification, x100).

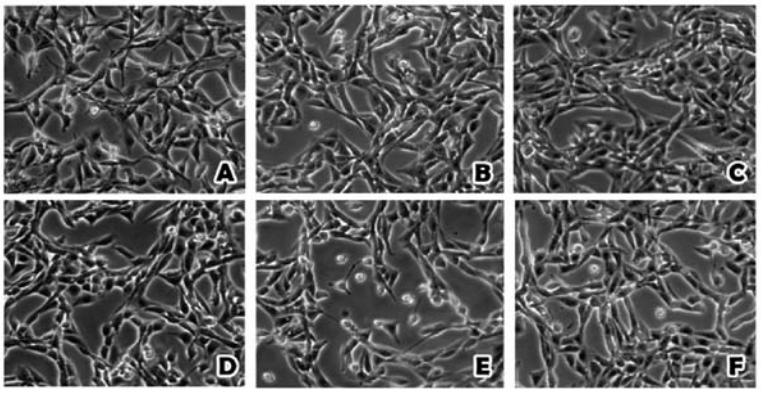

Figure 2. Effect of arecoline on the cell morphology of Hel cells. Concentrations of arecoline: (A) 0 μg/ml, (B) 25 μg/ml, (C) 50 μg/ml, (D) 75 μg/ml, (E) 100 μg/ml and (F) 125 μg/ml (magnification, x100).

with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the specific signals were visualized using ECL detection system. Antibodies against cyclin D1, CDK4, CDK2, E2F1, cyclin B, CDC2, cyclin A and cyclin E were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Statistical analysis. Differences in nonparametric variables were analyzed by the Fisher's exact test using the EPI software (EPI Info, version 3.2.2, www.CDC.gov/epiinfo/). Differences in the quantitative variables between groups were analyzed by the Student's t-test using SPSS 11.0 program (SPSS, Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant result.

Results

Effect of arecoline on HaCaT cell morphology. The HaCaT cell morphology was significantly altered following treatment of arecoline at different concentrations after 48 h. The outlines of the HaCaT cells were clear when treated with 0 and 25 μg/ml, but when the concentration of arecoline increased to 50 μg/ml, the boundaries of the HaCaT cells became indistinct and some of the cells were dead. There was massive cell death and a lack of boundaries between the cells when the concentration of arecoline was increased to 75, 100 and 125 μg/ml (Fig. 1). In contrast, the Hel cells were less affected by arecoline treatment. Only when the concentration of arecoline was increased to 100 and 125 μg/ml, did the Hel cell morphology begin to change slightly (Fig. 2).

Arecoline suppresses HaCaT cell proliferation. To further test whether arecoline modulates HaCaT cell growth, HaCaT and Hel cells were treated with 0, 25, 50, 75, 100 and 125 μg/ml arecoline, and their spontaneous proliferation was determined by MTT assays. Treatment with arecoline significantly inhibited the proliferation of HaCaT cells (Fig. 3A), but only slightly reduced Hel cell growth *in vitro* (Fig. 3B). Therefore, treatment with arecoline inhibited the proliferation of epithelial cells, but not fibroblasts *in vitro*.

Arecoline induces the cell cycle arrest of HaCaT cells. Inhibition of cell proliferation usually is mediated by inducing cell cycle arrest. To test this possibility, HaCaT and Hel cells were treated with 0, 25, 50, 75, 100 and 125 μg/ml arecoline and the cell cycle distribution was determined by FACS analysis (Tables II and III). In comparison to the control cells without arecoline treatment, following treatment with ≥75 μg/ml

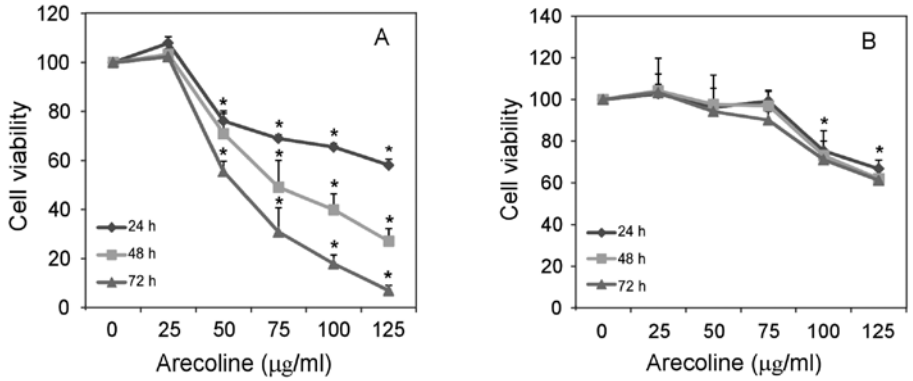

Figure 3. (A) Arecoline inhibits epithelial cell proliferation (HaCaT). (B) Effect of arecoline treatment on the viability of fibroblast cells (Hel).

Table II. Flow cytometric analysis of HaCaT cells following treatment with different concentrations of arecoline.

	Phase of the cell cycle[a]		
Treatment	G_0-G_1 (%)	S (%)	G_2-M (%)
Arecoline (μg/ml)			
0	49.02	32.05	18.93
25	51.06	31.94	17.00
50	53.62	30.69	15.69
75	73.00	20.50	6.50
100	91.23	5.61	3.16
125	89.67	5.13	5.20

[a]Numbers indicate the percentage of cells in the different phases of the cell cycle.

Table III. Flow cytometric analysis of Hel cells following treatment with different concentrations of arecoline.

	Phase of the cell cycle[a]		
Treatment	G_0-G_1 (%)	S (%)	G_2-M (%)
Arecoline (μg/ml)			
0	52.52	33.35	14.13
25	49.09	34.02	16.89
50	50.04	30.17	19.79
75	51.75	30.21	18.04
100	54.02	31.64	14.34
125	54.86	30.92	14.22

[a]Numbers indicate the percentage of cells in the different phases of the cell cycle.

arecoline a higher percentage of HaCaT cells remained at the G_0/G_1 phase of the cell cycle (from 49.02 to 73.00%) at 12 h, accompanied by a reduced percentage of cells in the S phase (from 32.05 to 20.50%). However, arecoline treatment did not significantly alter Hel cell cycling even after treatment with arecoline for 12 h. Hence, these data demonstrate that arecoline treatment induces cell cycle arrest by inhibiting the G_1 to S phase transition of the cell cycle in epithelial cells *in vitro*.

Arecoline regulates the transcription level of cell cycle regulatory molecules in the HaCaT epithelial cells. To reveal the possible mechanism of the effect of arecoline on epithelial cells *in vitro*, the levels of mRNA transcripts of several cell cycle regulatory molecules (CDK2, CDK4, cyclin A, cyclin B, cyclin D1, cyclin E, E2F1, CDC2) were determined by semi-quantitative RT-PCR. Cyclin D1 mRNA expression was downregulated following arecoline stimulation, while

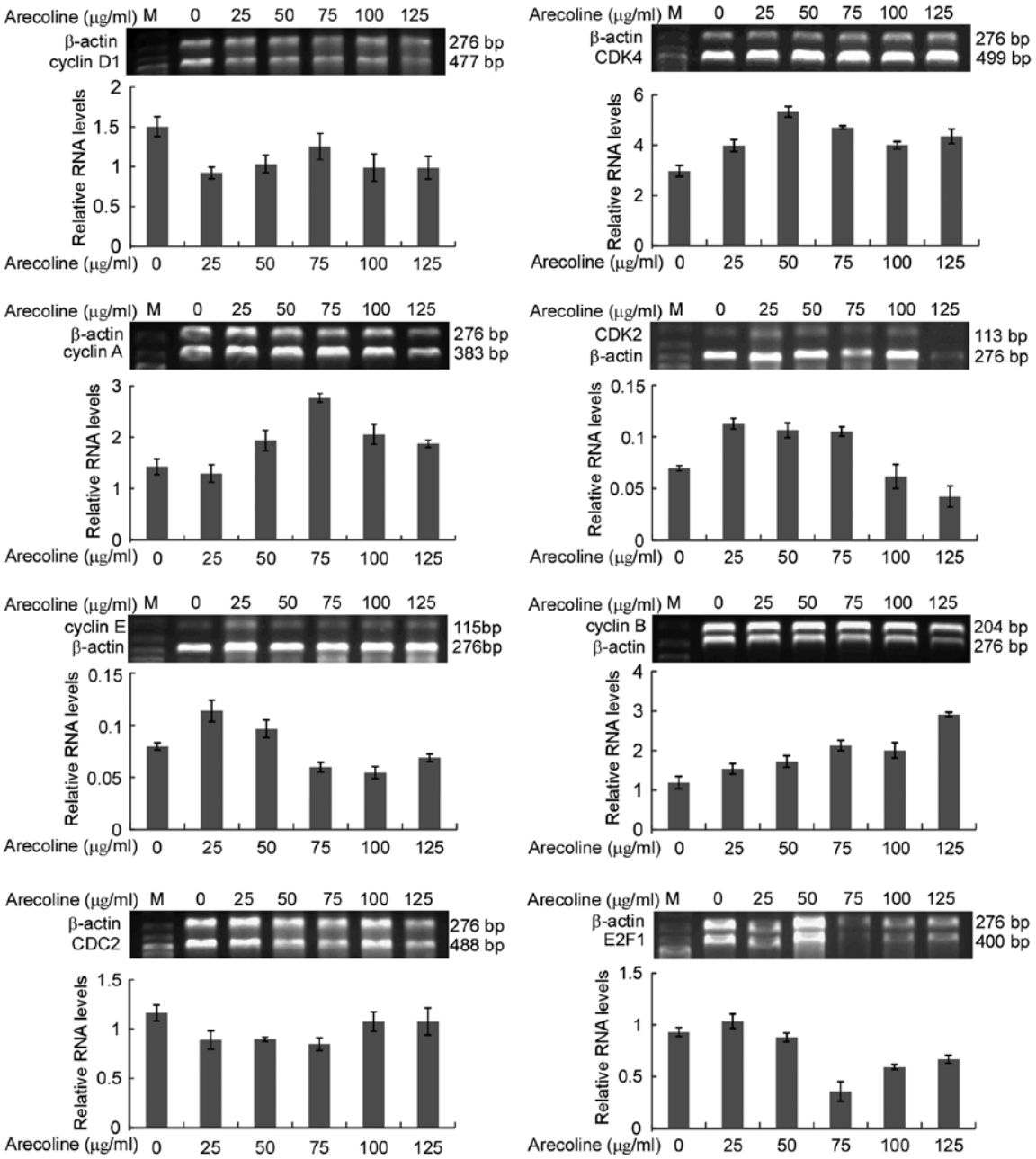

Figure 4. Arecoline regulates the transcription level of cell cycle regulatory molecules in the epithelial HaCaT cells.

there was no significant concentration gradient dependence. The mRNA expression of cyclin B was upregulated. Low concentrations of arecoline stimulated CDK4 expression. However, its expression was suppressed following treatment with high concentrations of arecoline. Cyclin A expression level was increased in the HaCaT cells following treatment with arecoline. Cyclin E expression level was decreased with an increase in arecoline concentrations. CDK2 had the same tendency as cyclin E. There was no significant difference in CDC2 levels (Fig. 4).

Arecoline regulates the protein levels of cell cycle regulatory molecules in the HaCaT epithelial cells. To confirm the change mechanism of cell cycle regulatory molecules, we tested the protein levels of CDK2, CDK4, cyclin A, cyclin B, cyclin D1, cyclin E, E2F1 and CDC2 by western blotting. The expression levels of cyclin D1, CDK4, CDK2 and E2F1 were significantly downregulated. Expression of cyclin A and cyclin E was slightly upregulated in HaCaT cells following arecoline stimulation. There was no significant difference in cyclin B and CDC2 expression (Fig. 5).

Discussion

Betel nut chewing is the most common cause of OSF. Arecoline is the main component of the betel nut, and it is associated with the occurrence and development of OSF through cytotoxicity, genotoxicity and DNA damage. Arecoline inhibits oral keratinocytes and vascular endothelial cell growth (17,18). However, using identical arecoline stimulation, OSF epithelial tissues

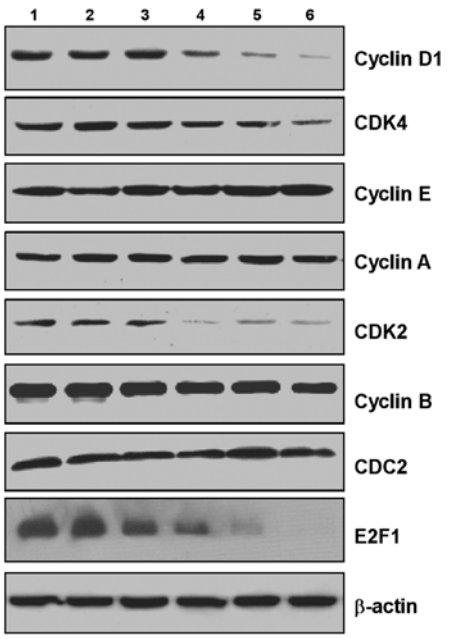

Figure 5. Arecoline affects the protein expression level of cell cycle regulatory molecules in HaCaT epithelial cells. Lane 1, 0 μg/ml arecoline; lane 2, 25 μg/ml; lane 3, 50 μg/ml; lane 4, 75 μg/ml; lane 5, 100 μg/ml; lane 6, 125 μg/ml.

became atrophic and presented vascular occlusion, and showed the beginning of the accumulation of fibroblasts and collagen fibers. Similar types of stimuli elicit differential responses in different cell types. In the present study, we studied the effects of arecoline on HaCaT epithelial cells and Hel fibroblasts.

Our data demonstrated the effects of arecoline on HaCaT cell morphology. The cell morphologies were significantly different dependent on the arecoline concentration after 48 h of treatment. In contrast, the Hel cells showed no effects following arecoline treatment. MTT assay revealed that arecoline suppressed HaCaT cell proliferation. Treatment with arecoline significantly inhibited the proliferation of HaCaT cells, but only slightly reduced Hel cell growth *in vitro*. Therefore, treatment with arecoline inhibited the proliferation of epithelial cells, but not fibroblasts, *in vitro*. Our results confirmed our hypothesis: different cells have differential responses to similar types of stimuli.

Cell cycle checkpoints are biochemical pathways that ensure the orderly and timely progression and completion of critical events, such as DNA replication and chromosome segregation (9,19). In the present study, we found that arecoline induced HaCaT cell cycle arrest. In comparison with control cells without arecoline treatment, following treatment with ≥75 μg/ml arecoline a higher percentage of HaCaT cells remained at the G_0/G_1 phase of the cell cycle, accompanied by a reduced percentage of cells in the S phase. However, arecoline treatment did not significantly alter Hel cell cycling. Huang *et al* (13) found that arecoline decreased interleukin-6 production and induced apoptosis and cell cycle arrest in human basal cell carcinoma cells. Arecoline, a major alkaloid of the areca nut, was found to inhibit p53, repress DNA repair, and trigger DNA damage response in human epithelial cells (21). Cyclin D1 expression and its possible regulation in chewing tobacco have been shown to mediate oral squamous cell carcinoma progression (22).

To reveal the possible mechanism of arecoline effect on epithelial cells *in vitro*, the levels of mRNA transcripts of cell cycle regulatory molecules were determined by semi-quantitative RT-PCR and western blotting. Short-term high-dose arecoline exerted little effect on Hel fibroblast cell growth, but significantly inhibited HaCaT epithelial cell growth and arrested HaCaT cells at the G_1/S phase, which was mainly through decreased protein expression and gene transcription of cyclin D1, CDK4, CDK2, E2F1, but had no obvious effects on cyclin B/CDC2 which is related to the G_2/M phase. Thus, epithelial atrophy in OSF is mainly attibuted to cell cycle arrest and imbalance of proliferation and apoptosis induced by arecoline. Studies have shown that expression of the transcription factor E2F1 was increased in tumors and proliferative diseases (11,17,18,23-25). When cells cross the G_1/S restriction point, the cell cycle path becomes irreversible with autonomy until the end of the cell cycle (26-29). Arecoline had no significant effect on fibroblasts in the G_1/S phase, which may explain the reasons for subcutaneous fibroblast proliferation-induced submucosa collagen accumulation (28,29).

In summary, arecoline inhibits HaCaT epithelial cell proliferation and survival, in a dose-dependent manner with cell cycle arrest in the G_1/S phase, while this observation is not obvious in Hel fibroblast cells. Arecoline downregulates the expression of G_1/S phase regulatory proteins cyclin D1, CDK4, CDK2 and E2F1 in HaCaT epithelial cells. Potentially, our findings may aid in the prevention of arecoline-associated human OSF.

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