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Effects of nicotine on proliferation, cell cycle, and differentiation in immortalized and malignant oral keratinocytes

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BACKGROUND: Numerous epidemiological studies have reported that tobacco smoking is a major risk factor for oral cancer, but relatively little is known about the effect of nicotine, a major product of cigarette smoking, on immortalized oral keratinocytes and cancer cells.
METHODS: We investigated the effects of nicotine on the growth and differentiation of immortalized human oral keratinocytes (IHOK), primary oral cancer cells (HN4), metastatic oral cancer cells (HN12), and human skin keratinocytes (HaCaT), in the monolayer and in the three-dimensional (3D) raft cultures using the MTT assay, Western blotting, and cell cycle analysis.
RESULTS: Nicotine inhibited the proliferation of immortalized and malignant keratinocytes in dose- and time-dependent manners as determined by MTT assay. The 3D organotypic culture showed that nicotine at high concentration (300 μM) inhibits epithelial maturation, surface keratinization, and decreased epithelial thickness. Flow cytometry showed that nicotine inhibited cell cycle progression by inducing G_0/G_1 arrest of HaCaT, IHOK, HN4, and HN12 cells without causing apoptosis. Nicotine treatment increased p21 expression in immortalized cells (HaCaT, IHOK) and oral cancer cells (HN4, HN12), but decreased pRb and p53 expression in oral cancer cells. Moreover, after high-dose nicotine treatment, the involucrin expression increased markedly in immortalized cells, but not in oral cancer cells.
CONCLUSIONS: We demonstrated that nicotine inhibits its growth through cell cycle arrest at G_0/G_1 phase probably by increasing the expression of $p21^{WAF1/CIP1}$. Nicotine also affects epithelial differentiation in immortalized and malignant oral keratinocytes. Malignant oral keratinocytes appear to be more resistant to the effects of nicotine on epithelial growth and differentiation as compared to the immortalized cells.
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Keywords: cell cycle; differentiation; growth; immortalized oral keratinocytes; nicotine; oral cancer; organotypic culture

Introduction

Oral cancer is the fifth most common malignancy worldwide and 90% of all oral malignancies are squamous cell carcinoma (SCC). The etiology of the disease is obscure, although epidemiologic studies have identified tobacco and alcohol use as the most important risk factors (1). It has been suggested that smoking influences the persistent cervical infection of oncogenic human papillomavirus (HPV), an additional risk factor for orogenital cancer (2–4). Furthermore, an adverse effect of smoking on the biologic behavior or response to the treatment of oral cancer cannot be discounted.

Nicotine, which is a major component of cigarette smoke, is one of a few natural liquid alkaloids. It is a colorless, volatile base ($pKa = 8.0–8.5$) that turns brown and produces the odor of tobacco on exposure to air. Nicotine is readily absorbed from the respiratory tract, buccal mucosa, and skin. The absorption and excretion of nicotine depends on the pH of the biological membrane or urine. The effects of nicotine on cellular function are dose dependent, vary with cell type, and primarily affect the central nervous system (5). Nicotine can also alter some cellular functions, such as cell growth, attachment, and matrix protein synthesis (6–8). Nicotine stimulates the initiation of DNA synthesis or the *in vitro* growth of smooth muscle cells (9), murine embryonic fibroblasts (10), and human embryonic diploid lung fibroblasts (11). Other report demonstrated

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the dose-dependent stimulatory effect of nicotine on epithelial cell growth and concluded that the selective mitogenic effect of nicotine was related to the increased risk of SCC (12). However, nicotine does not always stimulate cell growth or proliferation. Nicotine inhibited the proliferation of human promyelocytic HL-60 leukemic cells and BALB/3T3 cells (13, 14).

Oral mucosal keratinocytes are the cells that first encounter tobacco components. Therefore, the tobacco-induced abnormal alteration of oral mucosal keratinocytes may contribute to the development of premalignant oral white lesions. Although a number of studies have characterized the direct effects of tobacco or its constituents on normal human oral keratinocytes (15–17) and fibroblasts (18), there are no comparative studies on the effects of nicotine on skin keratinocytes vs. oral keratinocytes and immortalized cells vs. oral cancer cells. Therefore, we evaluated here the nicotine-induced changes in growth, cell cycle, and epithelial differentiation in human skin keratinocytes (HaCaT), oral immortalized keratinocytes (IHOK) and oral SCC cell lines (HN4, HN12). In addition, we utilized organotypic three-dimensional (3D) cultures of stratified epithelium as an *in vitro* tissue model to study the effect of nicotine (3, 19).

Materials and methods

Cell culture

HPV-immortalized human oral keratinocytes (IHOK) were derived by transfecting normal human gingival epithelial cells with PLXSN vector containing the E6/E7 open reading frames of HPV type 16, following methods previously described (3). Stably transfected cells were selected using G418. The immortalized oral keratinocytes were cultured in the keratinocyte growth medium (KGM, Gibco, Grand Islands, NY, USA) supplemented with 2 ml of bovine pituitary extract (13 mg/ml), 0.5 ml each of hydrocortisone (0.5 mg/ml), human epidermal growth factor (0.5 μg/ml), insulin (5 mg/ml), epinephrine (0.5 mg/ml), transferrin (10 mg/ml), triiodothyronine (6.5 μg/ml), and GA-1000 and 0.05 mM $CaCl_2$.

HaCaT, HN4 (=HNSCC4), and HN12 (=HNSCC12) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biofluid, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, NY, USA) with 100 U/ml penicillin and 100 U/ml streptomycin (Life Technologies, Gaithersburg, MD, USA). Although HaCaT cells are immortalized and are genetically abnormal (20), they retain many features of keratinocyte differentiation and represent a skin keratinocyte model. Cell line HN4 from a primary $T_3N_0M_0$ carcinoma of the mouth floor and HN12 from metastatic carcinoma of the oral cavity (21) was derived in the laboratory of Dr John F. Ensley (Wayne State University). All the cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were dissociated with 0.25% trypsin just before transfer for experiments and were counted using a hemocytometer.

The DMEM, KGM medium (Clonetics), FBS, and other tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). Anti-p16, p21, p53, pRb, CK13, CK19, and involucrin antibody were purchased from Santa Cruz (CA, USA). Nicotine and all other chemicals were obtained from Sigma Chemical (St. Louis, MO, USA).

MTT assay

Viable cells were detected using MTT dye, which forms blue formazan crystals that are reduced by mitochondrial dehydrogenase present in living cells. Briefly, 2×10^4 cells were seeded in a 96-well plate and cultured overnight for cell attachment. Serial dilutions of nicotine were added, and cells were treated for 1, 3, and 5 days. After treatment, 50 μl of MTT solution (2 mg/ml in PBS) were added to each well and incubated for 4 h. The plates were then centrifuged at 200 *g* for 10 min and the supernatant was discarded. To each well, 50 μl of DMSO were added. The plates were then shaken until the crystals had dissolved. Reduced MTT was then measured spectrophotometrically in a dual beam microtiter plate reader at 570 nm. The nicotine concentration required to inhibit cell growth by 50% (IC_{50}) was determined by interpolating the dose-response curves.

Raft culture of epithelial cells and nicotine treatment

Dermal equivalent collagen gels were prepared as previously described (22) using type I collagen (Nitta Gelatin, Osaka, Japan). Confluent monolayer cells were trypsinized using 0.5% trypsin/EDTA (Gibco, Grand Island, NY, USA), and seeded on a freshly prepared gel containing type I collagen matrix and primary human gingival fibroblasts. The gel was kept submerged in a Millicell (Becton Dickinson, San Jose, CA, USA) for 5 days, and fed with fresh medium every 24 h. Then, the gel was lifted to the liquid/air interface in order to have the nutrients supplied vertically from bottom to top and to induce keratinocyte differentiation mimicking a stratified epithelium. The raft culture was maintained with or without nicotine treatment for 14 days, and the organotypic tissue was fixed entirely in 10% buffered formalin, sectioned in 4 μm thicknesses, and stained for microscopic analysis and photography.

Cell cycle analysis

Cells (2×10^5) cultured with or without nicotine for 3 days were harvested, fixed with 75% ethanol at 4°C for 2 h, and then treated with 0.25 mg/ml RNase A (Sigma Chemical) at 37°C for 1 h. After washing, the cells were stained with 500 μg/ml propidium iodide (PI, Sigma Chemical) at room temperature for 10 min. Cell cycle analysis was performed on a EPICS-XL flow cytometer (Coulter, FL, USA) using the cell fit analysis program for the staining profile of viable cells.

Western blot analysis

Protein samples (50 μg) were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and then separated using 8–15% SDS-gels.

After electrophoresis, the proteins were transferred to nylon membranes. The membranes were blocked in 5%

dry milk (1 h), rinsed, and incubated with the primary antibodies (diluted 1:500–1:1000) followed by the secondary antibodies in TBS for 1 h at room temperature. Finally, each protein was detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, USA).

Results

Effects of nicotine on cell proliferation

In order to compare the effects of nicotine on the cell growth of skin (HaCaT) and oral keratinocytes (IHOK), exponentially growing cells were treated with nicotine and the cell viability was evaualted by MTT assay. The growth of HaCaT cells was inhibited in a time- and dose-dependent fashion, at concentrations from 60 to 600 μM and for 3 and 5 days of culture. At 600 μM nicotine, about 10% of the HaCaT cells survived at 5 days of cultivation, while at 6 μM nicotine, over 70% survived (Fig. 1a). The proliferation of IHOK cell was decreased significantly at nicotine concentrations of 60–600 μM after 3 and 5 days in a dose dependent manner (Fig. 1b). The maximum inhibition of IHOK cell proliferation was obtained at the concentration of 600 μM (~99%) at day 5 of cultures. The growth of HaCaT cells was not significantly different from IHOK cells in the presence of nicotine as determined by MTT. Overall, these data suggest that there is no significant difference in the nicotine-induced cell growth inhibition between the immortalized skin keratinocytes and the immortalized oral keratinocytes.

We next investigated the effect of nicotine on the growth of HN4 (primary oral cancer cells) and HN12 (metastatic oral cancer cells), and compared with IHOK (immortalized cells). Growth inhibition was observed with both HN4 and HN12 cells in the presence of nicotine. However, these cancer cells appeared to be less sensitive to nicotine than the immortalized keratinocytes. Of the two cancer cells, the metastatic cell line HN12 was more resistant than the primary cancer line HN4.

The concentration of nicotine causing 50% inhibition of cell growth (IC_{50}) was calculated after 1, 3, and 5 days of exposure (Fig. 2). The IC_{50} of HaCaT and HN12 cells was higher than that of IHOK, suggesting both HaCaT and HN12 cells are more resistant to the nicotine-induced toxicity than IHOK cells.

The effects of nicotine on the organotypic cultures

To establish the nicotine concentration to use in organotypic culture experiments, we conducted preliminary studies of HaCaT cells using nicotine concentrations from 0.6 to 600 μM. While no morphologic alteration was seen at 0.6 and 6 μM, most of the epithelial architecture was lost at 600 μM (data not shown). Epithelial growth alteration was detected in response to nicotine in the range from 60 to 300 μM. An amount of 300 μM was studied because this concentration induced growth inhibition reproducibly in almost 50% of the cells by 3 days of culture.

Organotypic culture of control HaCaT cells resulted in stratification with well-preserved morphologic differentiation. Generally, at least six layers developed, with good cellular polarity of differentiation, showing distinct keratin layer (Fig. 3a). By contrast, nicotine-treated HaCaT cells produced only one or two cell layers, with less keratinization and stratification than the untreated control (Fig. 3b). Control IHOK cells also produced typical stratification and multiple layers, while nicotine-treated IHOK cells stratified prematurely and showed reduced thickness.

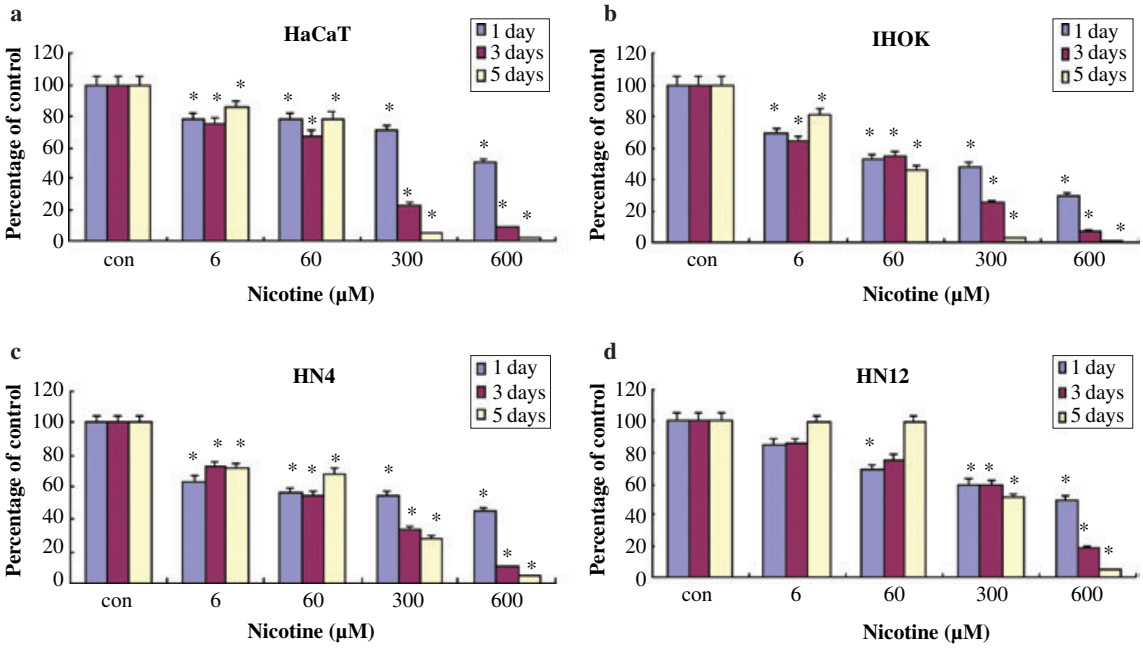

Figure 1 Effects of nicotine on cell viability on HaCaT (a), immortalized human oral keratinocyte (b), primary oral cancer (HN4) (c), and metastatic oral cancer (HN12) (d) cells as measured by MTT assay. Each points and bar represent a mean ± SD. $^*P < 0.05$ vs. controls.

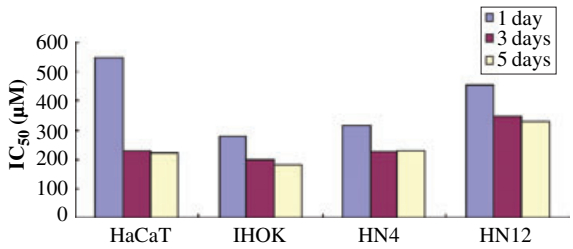

Figure 2 Nicotine concentration for 50% growth inhibition (IC_{50}) in immortalized and malignant oral keratinocytes.

Oral cancer cells (HN4, HN12) reconstituted in 3D organotypic culture revealed the cellular malignancies of hyperchromatism and pleomorphism in the microsections. Nicotine-treated HN4 and HN12 cells reconstituted in 3D organotypic culture showed less epithelial maturation, and decreased epithelial thickness and surface keratinization as compared to the untreated cells. Light microscopy showed that keratinocyte growth was greatly retarded in the cells exposed to 300 μM nicotine, as shown by decreased keratinization, and a reduced number of cell layers, regardless of keratinocyte type (Fig. 3b).

Changes in the cell cycle distribution with nicotine treatment

To understand whether the nicotine-induced growth inhibition of immortalized and malignant oral keratinocytes cells was caused by cell cycle arrest, we examined the cell cycle distribution using flow cytometry. Cells were cultured with or without 300 μM nicotine for 3 days, washed and stained with PI, and then the cell cycle was analyzed. As shown in Table 1, 58.81% of control HaCaT cells were at G_0/G_1 phase, and 16.24%, 9.26%, and 12.62% were at S, G_2/M, and sub-G_1 phase, respectively. After 3 days of nicotine treatment in HaCaT cells, G_0/G_1 phase cells increased to 74.41% while the proportion of S phase cells decreased to 6.57%.

The IHOK cells treated with nicotine showed a pattern of cell cycle change similar to that of HaCaT cells. With IHOK cells, however, the changes in G_1 and S phase were less profound than with HaCaT cells. The fraction of sub-G_1 cells did not change with the nicotine concentration or culture time in HaCaT and IHOK cells. Both HN4 and HN12 cells also showed similar pattern as in HaCat and IHOK. The percent of cells in G_0/G_1 phase was increased after nicotine treatment, while that in the S phase was decreased, but there were no significant differences in G_2/M and sub-G_1 phase for the experimental groups of immortalized and malignant keratinocytes. These results suggest that nicotine inhibits proliferation of cells by arresting cells in G_1 phase.

Effects of nicotine on the intracellular levels of cell cycle regulatory proteins

The levels of p53 protein were reduced in nicotine-treated HaCaT cells and also in IHOK, whereas the levels of p21 were increased compared to that of

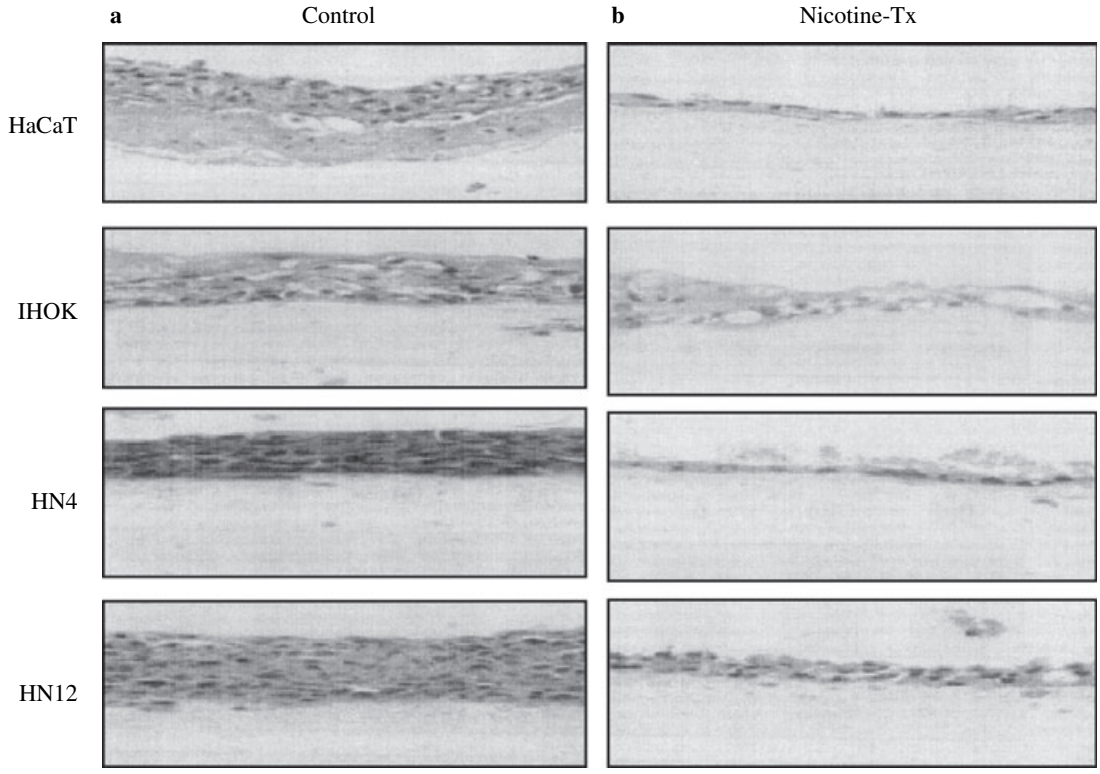

Figure 3 Organotypic cultures of immortalized and malignant oral keratinocytes cells treated with or without 300 μM nicotine for 2 weeks (hematoxylin and eosin stain). (a) Control epithelial cells exhibiting usual strafication and multiple layers of cells (×200). (b) Same cells treated with nicotine. These cells showed poor stratification and less organized cell layers (×200).

Table 1 The effect of nicotine on cell cycle distribution in immortalized and malignant oral keratinocytes

Cell lines	*Nicotine (μM)*	*Sub-G_0/G_1 (%)*	*G_0/G_1 (%)*	*S (%)*	*G_2/M (%)*
HaCaT	0	12.62 ± 9.17	58.81 ± 8.72	16.24 ± 0.77	9.26 ± 0.70
	300	10.37 ± 2.12	74.41 ± 0.84	6.57 ± 0.52	7.44 ± 0.09
IHOK	0	14.01 ± 1.28	53.21 ± 0.38	17.82 ± 0.01	8.73 ± 0.2
	300	14.87 ± 0.54	57.07 ± 0.77	14.55 ± 0.24	9.09 ± 1.01
HN4	0	11.00 ± 0.50	45.43 ± 0.20	25.39 ± 0.28	10.41 ± 0.16
	300	11.82 ± 2.24	49.10 ± 3.41	22.85 ± 0.11	12.78 ± 0.53
HN12	0	12.95 ± 1.25	48.53 ± 1.88	24.56 ± 0.56	11.56 ± 0.26
	300	14.54 ± 5.16	52.99 ± 1.06	19.44 ± 2.46	10.01 ± 2.09

These data are a mean ± SD of three independent experiments.

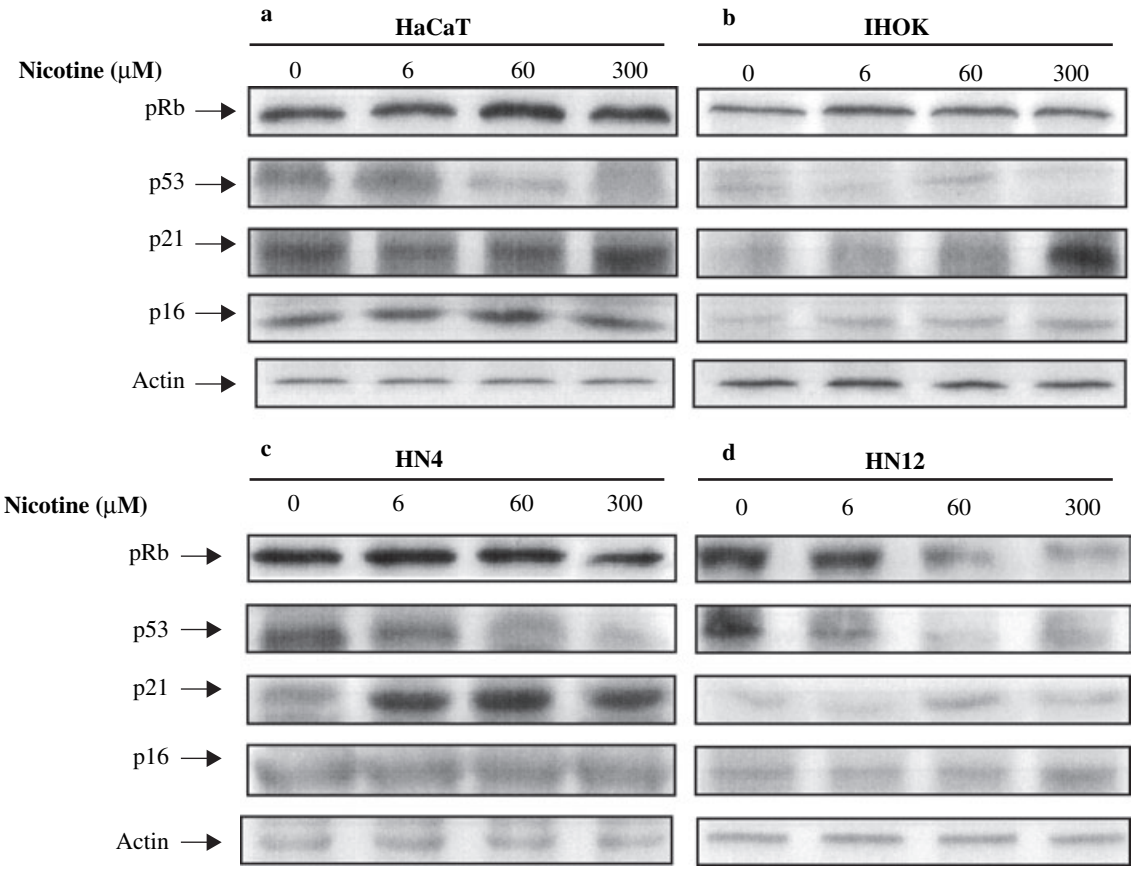

Figure 4 Western blot analysis of cell cycle regulatory protein expression of p16, p21, p53, and pRb in HaCaT (a), IHOK (b), HN4 (c), and HN12 (d) cells cultured without or with different concentration of nicotine for 3 days. The protein fraction was extracted, electrophoresed, transferred to membrane and blotted with respective antibodies (lane 1: control; lane 2: 6 μM; lane 3: 60 μM; lane 4: 300 μM nicotine treated). These data are representative of three independent experiments.

controls (Fig. 4a). Little changes in p16 were observed. These results suggest that the decrease in cell proliferation and lengthened Gap phase (G_1) by nicotine treatment are due to the increased expression of p21 and that induction of p21 occurred by a p53-independent pathway the nicotine-treated immortalized keratinocytes.

In oral cancer cells (HN4 and HN12 cells), the levels of p53 and pRb in the test group were lower than in controls, whereas p21 was increased compared with the control. Little difference in p16 level was observed. This pattern of cell cycle regulatory protein expression is similar to that of HaCaT cells (Fig. 4c,d). Collectively, these results suggest that nicotine induces cell cycle arrest in immortalized and malignant keratinocytes by increasing p21 expression.

Western blot analysis of the intracellular levels of epithelial differentiation markers

We examined the expression of involucrin, a protein precursor of the epidermal cornified envelope, and cytokeratins, epithelial specific proteins, after treating

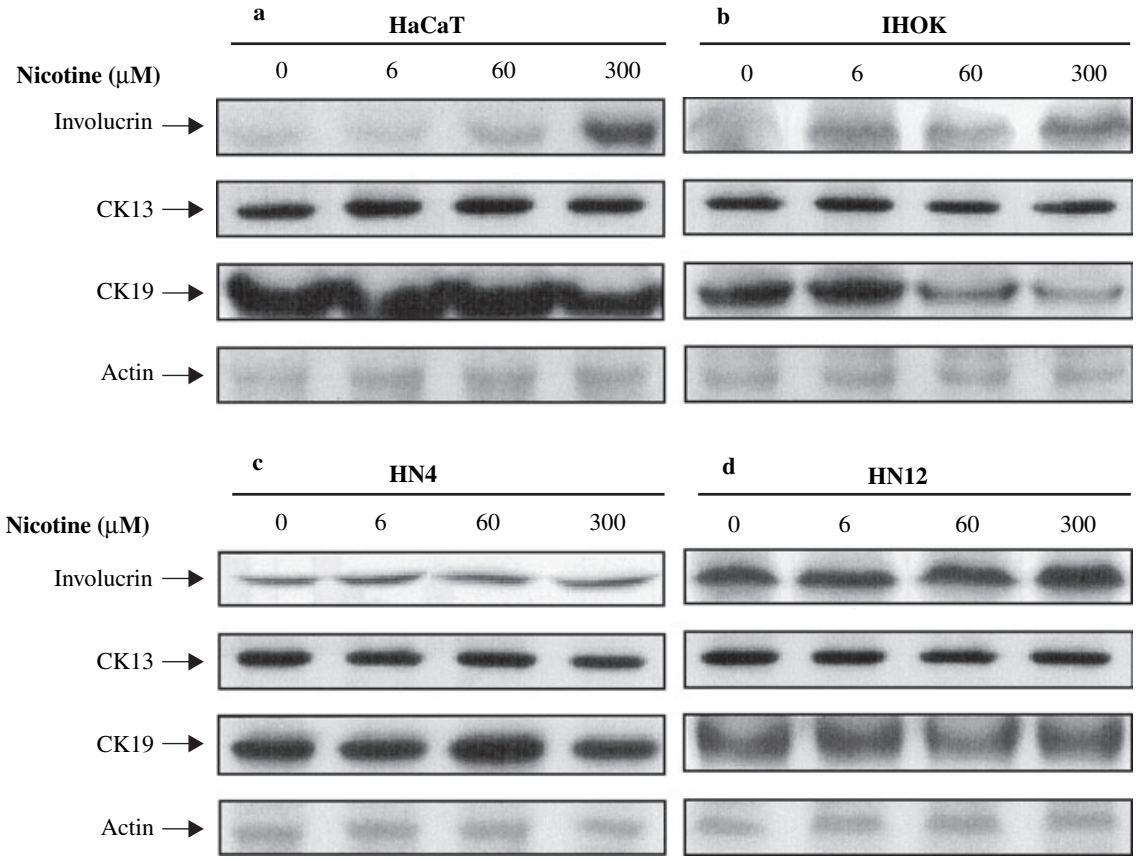

Figure 5 Western blot analysis of keratinocyte differentiation marker expression of involucrin, cytokeratin (CK) 13, and CK 19 in HaCaT (a), IHOK (b), HN4 (c), and HN12 (d) cells cultured without or with different concentration of nicotine and same procedure as described in the legend to Fig. 4 was performed (lane 1: control; lane 2: 6 μM; lane 3: 60 μM; lane 4: 300 μM nicotine treated). These data are representative of three independent experiments.

immortalized and malignant keratinocytes with nicotine for 3 days. High-dose nicotine increased the expression of involucrin, especially in immortalized cells (HaCaT, IHOK), but no change in oral cancer cells (HN4, HN12) (Fig. 5a–d).

As shown in Fig. 5a and b, there were no major differences in CK13 expression in immortalized and malignant oral keratinocytes cells treated with nicotine. By contrast, following nicotine treatment, the expression of cytokeratin 19 decreased in immortalized cells (HaCaT, IHOK), but not in the malignant cells (HN4, HN12).

Collectively, low-dose nicotine did not alter the expression of the keratinocyte differentiation marker, while high-dose nicotine significantly enhanced the expression of involucrin in immortalized keratinocytes, but not in malignant keratinocytes. These results suggested that HN4 and HN12 cells (malignant oral keratinocytes) were more resistant to nicotine-induced differentiation than were the HaCaT and IHOK cells (immortalized keratinocytes).

Discussion

Oral cancer has a strong etiologic association with smoking. Although previous studies have shown the effects of nicotine in leukemia, lung, and cervical cancer cells (12, 13, 23, 24), the effects of nicotine in oral precancerous and cancer cells were unknown. Therefore, we investigated the influence of nicotine on proliferation, the cell cycle, and differentiation in HPV16 E6/E7-immortalized human oral keratinocytes (IHOK), immortalized human skin keratinocytes (HaCaT), and primary (HN4) and metastatic oral cancer cells (HN12).

We found no significant difference in the nicotine-induced growth inhibition in HaCaT (skin) and IHOK (oral immortalized keratinocytes) cells. In contrast, Kwon et al. (25) reported that epidermal keratinocytes isolated from foreskin are slightly more susceptible to the cytotoxic effects of nicotine than those isolated from oral mucosa. The reason for this conflict in the effects of nicotine on skin and mucosal equivalents is unclear, but may reflect the differences between the primary keratinocytes and the immortalized keratinocytes. The intrinsically predetermined region-specific phenotypes of keratinocytes from the buccal mucosa and skin may contribute to the pharmacological response to nicotine (22).

We observed an inhibitory effect of nicotine on human oral immortalized and malignant keratinocyte proliferation after treatment at 60 μM nicotine. At low

concentrations (6 μM), nicotine barely impaired the proliferation of the immortalized and malignant oral keratinocyte cell lines tested. The nicotine effect was dose-dependent, with maximal inhibition with 600 μM nicotine after 5 days exposure. Other investigators also reported the effects of nicotine in other cell types. For example, nicotine stimulated the growth (110% of control) of primary human endothelial cells at 50 ng/ml (26) and of another lung cancer line with neuroendocrine features (24) at 1 μM. By contrast, no effect was noted on the growth of several human lung cancer cell lines at concentrations ranging from 16.2 pg/ml to 162 ng/ml (0.1 nM–1 μM) (23). In another study, nicotine impaired the growth of HeLa cells at concentrations exceeding 100 μg/ml, while lower concentrations had no effect on cell growth (27). These differences of nicotine effects most likely result from differences in the cell culture conditions, the cell model used, the degree of cell confluence, the concentration of nicotine studied, or interactions of nicotine or its metabolites.

As the skin and oral mucosa are composed of stratified squamous epithelium, we believe that a 3D culture method is an adequate model for investigating the pharmacological influence of drugs such as nicotine on the morphology, function, and differentiation of keratinocytes *in vitro* (25). We showed that the immortalized and malignant keratinocytes reconstituted in 3D organotypic culture treated with nicotine showed less epithelial maturation, and decreased epithelial thickness and surface keratinization, than control cells. Our 3D culture study showed that relatively long-term treatment with nicotine for 2 weeks completely disrupted growth and differentiation.

At nicotine concentrations in the range reported in the saliva of smokers (28, 29), the growth and differentiation of oral immortalized and malignant keratinocytes were inhibited. Several *in vitro* experiments have studied the effects of nicotine on different functions of periodontal tissue cells. Although the results are conflicting, depending on the nicotine concentration and exposure time, all the studies concluded that nicotine is capable of altering the host response by inhibiting major functions of periodontal cells, such as proliferation, protein synthesis, alkaline phosphates activity, collagen production, and chemotaxis (30–34).

To understand whether the nitotine induced growth inhibition was caused by cell cycle arrest, we analyzed the DNA content of cells using PI. This demonstrated that nicotine inhibits cell cycle progression by inducing the G_1 arrest of HaCaT, IHOK, HN4, and HN12 cells.

To investigate the mechanism by which nicotine inhibits the cell cycle progression of immortalized and malignant oral keratinocytes, we examined which proteins involved in regulating the cell cycle were affected. We found that, levels of p53 and pRb in oral cancer cells (HN4 and HN12) were reduced in the test group, whereas the level of p21 was increased in pre-malignant and malignant kerationocytes. The nicotine induced cell cycle arrest in immortalized and malignant keratinocytes was probably caused by increased p21 expression.

It was reported that nicotine enhanced the expression of the differentiation-specific proteins and induced the keratinization of oral mucosal and epidermal keratinocytes (25). Morphologically and biochemically, long-term treatment with nicotine for 2 weeks, at concentrations of 10^{-6}–10^{-4} M (0.162–16.2 μg/ml), enhanced the expression of differentiation markers of oral mucosal keratinocytes, such as CK13, involucrin, and profilaggrin/filaggrin (25). These findings confirm and extend the results of Grando et al. (35, 36) and Theilig et al. (29).

We found that at a high dose (300 μM), nicotine induced significant expression of involucrin in immortalized keratinocytes (HaCaT, IHOK), but not in the oral cancer cells. The expression of CK19 was decreased in pre-malignant cells but was not altered in oral cancer cells. These results indicate that oral cancer cells are more resistant to the nicotine effects than the immortalized keratinocytes.

In summary, nicotine treatment of immortalized (IHOK and HaCaT) and malignant (HN4 and HN12) keratinocytes markedly inhibits the proliferation of epithelial cells, retarding cell cycle progression. The impaired epithelial cell growth and differentiation in immortalized keratinocytes caused by nicotine may contribute to the transformation of pre-cancerous cells in chronic tobacco users. Obviously, more detailed studies of the mechanism of action of nicotine are necessary before the true clinical actions of nicotine in oral cancer progression can be determined.

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