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Regulation of extracellular signal-regulated protein kinase signaling in human osteosarcoma cells stimulated with nicotine

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Background: Cigarette smoking is a major risk factor in the development and further progression of periodontal diseases. Currently, there is limited information on the regulation of mitogen-activated protein kinases (MAPK) expression in smoking-associated periodontal disease.

Objectives: The aim of the present study was to investigate the effects of nicotine on the expression of MAPKs in human osteosarcoma cell line U2OS cells. Furthermore, various pharmacological agents were added to search the possible regulation mechanisms on nicotine-induced MAPKs expression.

Methods: Cytotoxicity and western blot assays were used to investigate the effects of U2OS cells exposed to nicotine. In addition, various pharmacological agents [NS-398, dexamethasome, 2-oxothiazolidine-4-carboxylic acid (OTZ), herbimycin A, and curcumin] were added to test how they modulated the effects of nicotine-induced MAPKs expression.

Results: Concentrations of nicotine higher than 5 mM demonstrated cytotoxicity to U2OS cells ($p < 0.05$). A nicotine concentration of 5 mM was found to induce extracellular signal-regulated kinase (ERK) phosphorylation in a time-dependent manner ($p < 0.05$). In addition, amounts of ERK protein were unaffected by nicotine during the same time interval. By contrast, nicotine has no effect on either c-Jun N-terminal kinase (JNK) or p38, respectively. In addition, NS-398, dexamethasone, OTZ, herbimycin A, and curcumin were found to inhibit the nicotine-induced ERK expression ($p < 0.05$).

Conclusions: The activation of ERK expression by nicotine suggests a potential role for nicotine in the pathogenesis of cigarette smoking-associated periodontal disease. In addition, nicotine-induced ERK expression was down-regulated by NS-398, dexamethasone, OTZ, herbimycin A, and curcumin.

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The relationship between cigarette smoking and periodontal disease is complex and controversial, but most studies strongly demonstrated that cigarette smoking is one of the most significant risk factors for the development and further progression of inflammatory periodontal disease (1, 2). In addition, response to conventional periodontal treatment has been documented as poor in cigarette smokers compared with non-smokers

(3). Clinical studies have also been observed in smokers after both soft tissue graft procedures (4) and in guided tissue regeneration procedures (5).

It has been proposed that tobacco products may produce their unwanted effects on the host locally due to direct exposure of the periodontal tissues to cigarette smoke. Nicotine is one of over 3800 potentially toxic components in cigarette smoke (6). Research has shown that nicotine can be detected on the root surfaces of periodontally involved teeth (7). Investigations using cell culture techniques have demonstrated that nicotine was a cytotoxic agent to fibroblasts derived from periodontium by inhibiting cell viability, attachment, proliferation, and matrix protein synthesis (7–10). However, a direct molecular role of nicotine in the periodontal disease has not yet been well defined. Our recent studies have shown that nicotine can induce c-*fos* gene expression in human periodontal ligament of fibroblasts (11) and cyclooxygenase-2 (COX-2) expression in gingival fibroblasts (12) *in vitro*. However, the mechanism behind the nicotine-induced expression of c-*fos*, COX-2 and other signal proteins still remains to be elucidated.

Protein phosphorylation at tyrosine residues is a key component in the regulation of eukaryotic cell growth, differentiation and other responses (13). The phosphotyrosine proteins have been linked to many cellular events, involving stimulation of mitogen-activated protein kinases (MAPKs). MAPKs are members of a serine/threonine kinase family that link receptor activation in the cell nucleus (14). MAPK family members are extracellular signal-regulated protein kinases (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK. ERK can be activated by some cell stress signals (15). One of the events required for stress-induced inflammation and carcinogenesis is the transcriptional elevation of c-*fos*, which in turn modulates downstream events including activation of MAPKs (16).

This study is designed to explore the effect of nicotine on MAPKs expression in human U2OS cells. Cytotoxicity and western blot assays were used to investigate the effects of U2OS cells exposed to nicotine. Furthermore, various pharmacological agents [NS-398, dexamethasome, 2-oxothiazolidine-4-carboxylic acid (OTZ), herbimycin A, and curcumin] were added to search the possible regulation mechanisms on nicotine-induced ERK expression.

Material and methods

All culture materials were obtained from Gibco (Grand Island, NY, USA). NS-398 (selective COX-2 inhibitor) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). Nicotine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dexamethasone [nuclear factor-κB (NF-κB) inhibitor], OTZ (precursor of glutathione), herbimycin A (tyrosine kinase inhibitor), and curcumin (AP-1 inhibitor) were purchased from Sigma (St. Louis, MO, USA). U0126 (a specific inhibitor of MEK 1/2 that inhibits ERK 1/2) was obtained from Promega (Madison, WI, USA).

Cell culture

U2OS cells (American Tissue Type Collection HTB 96), derived from human osteogenic sarcoma, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml of streptomycin, and 100 mg/ml of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2 (17). Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1 : 4 splits every third day.

Cytotoxicity assay

Cytotoxicity of nicotine was measured by a colorimetric assay using MTT dye (18). MTT solution was prepared in 5 mg/ml of phosphate-buffer saline just before use and filtered through a 0.22 μm filter. U2OS cells were seeded at 5×10^4 cells/well into 96-well culture plates and incubated to attach for 24 h. Cell was treated with various concentrations of nicotine (0–20 mM) and 10 μl of MTT solution was added to each well for 4 h. On termination, all the medium was discarded by inverting and tapping the plates and 100 μl of dimethyl sulfoxide was added to each well. The spectrophotometric absorbance at 540 nm was then measured by an enzyme-linked immunosorbent assay (ELISA) reader (Hitachi, U2000, Tokyo, Japan). The percentage of the dehydrogenase activity at each concentration, compared with that of the control, was calculated from the absorbance values.

MAPKs expression analysis

Nearly confluent monolayers of U2OS cells were washed with serum-free Dulbecco's modified Eagle's medium for 4 h and immediately thereafter exposed at the indicated incubation times to nicotine. The viability of cells exposed to these elutes were in general cytostatic according to the MTT assay. Cell lysates were collected at 1, 2, 4, 8, and 24 h. Subsequently, various pharmacological agents without cytotoxic concentrations were also added to wells to test their regulation effects during a 4 h incubation period. The final concentrations of NS-398, dexamethasone, OTZ, herbimycin A, and curcumin used in this study were 0.1 mM, 2 μM, 0.1 mM, 0.1 mM, and 0.05 mM, respectively.

Western blot

For western blot analysis, cell lyates were collected as described previously (19, 20). Briefly, cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM *N*-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000 *g* at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standard. Equivalent amounts of total protein per sample of cell extracts were run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The

membranes were blocked with phosphate-buffered saline containing 3% bovine serum albumin for 2 h, rinsed, and then incubated with primary antibodies anti-ERK (1 : 1000), anti-phospho-ERK (1 : 1000), anti-JNK (1 : 1000), anti-phospho-JNK (1 : 1000), anti-p38 MAPK (1 : 1000), and anti-phospho-p38 MAPK (1 : 1000) in phosphate-buffered saline containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1 : 1000 in the same buffer, washed again as described above and treated with 1 : 1000 streptavidin–peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using diaminobenzidine (Zymed Laboratories, Inc., South San Francisco, CA, USA). The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000, Alpha Innotech Corp., San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Statistical analysis

Three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test and a value of $p < 0.05$ was considered statistically significant.

Results

Nicotine demonstrated a cytotoxic effect on U2OS cells (Fig. 1). Nicotine reduced the activity of dehydrogenase of cells over a 4 h culture period in a dose-dependent manner ($p < 0.05$). However, the nicotine concentrations lower than 5 mM were shown cytostatic to cells.

Time-course experiments indicated that 5 mM nicotine induced ERK phosphorylation in a time-dependent manner, with maximal activity appearing 4 h after initiating exposure to nicotine (Fig. 2). In addition, amounts of ERK protein were unaffected by nicotine during the same time interval. By contrast, nicotine had no effect on either JNK or p38, respectively (Fig. 2).

The quantitative measurement was made by the AlphaImager 2000 (Fig. 3). The levels of the p-ERK activity increased about 2.2 and 3.6-fold after exposure to 5 mM nicotine for 1 and 4 h, respectively ($p < 0.05$). U0126 was used to block the activation of ERK. Treatment of U2OS cells with 23 μM U0126 decreased nicotine-mediated phosphorylation of ERK (Fig. 4). However, U0126 did not alter the total amounts of ERK protein.

Pharmacological agents without cytotoxic concentrations (0.1 mM NS-398, 2 μM dexamethasome, 0.1 mM OTZ, 0.1 mM herbimycin A, and 0.05 mM curcumin) were added to search the possible regulation

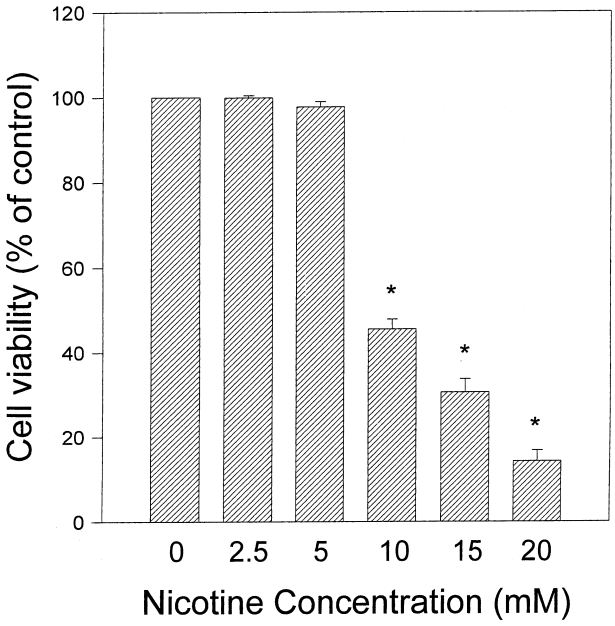

Fig. 1. Cytotoxicity of various concentrations of nicotine on U2OS cells as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Each point and bar represent a mean ± SD. *Denotes significant differences from control values with $p < 0.05$.

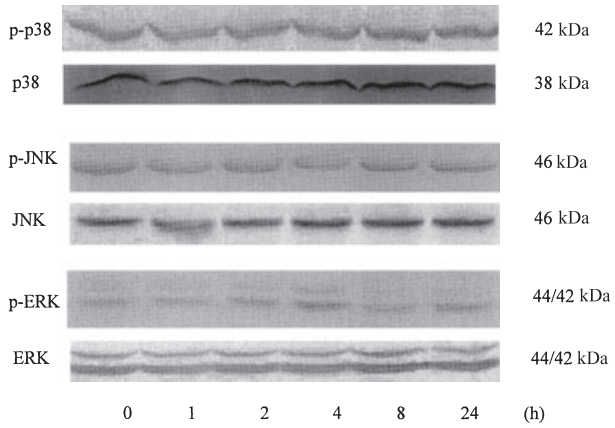

Fig. 2. Kinetics of mitogen-activated protein kinases (MAPKs) expression in U2OS cells exposed to 5 mM nicotine for 0, 1, 2, 4, 8, and 24 h, respectively. Phosphorylated and unphosphorylated MAPKs were detected by western blot analysis. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

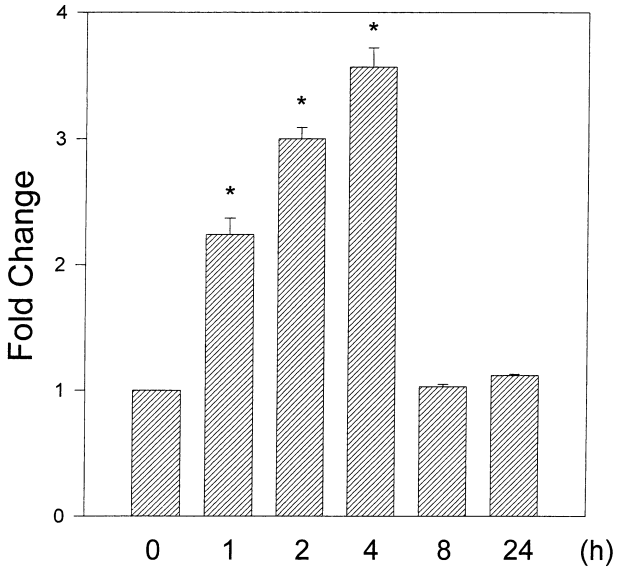

Fig. 3. Levels of phosphorylated extracellular signal-regulated kinase (p-ERK) treated with nicotine were measured by AlphaImager 2000. *Represents significant difference from control values with $p < 0.05$.

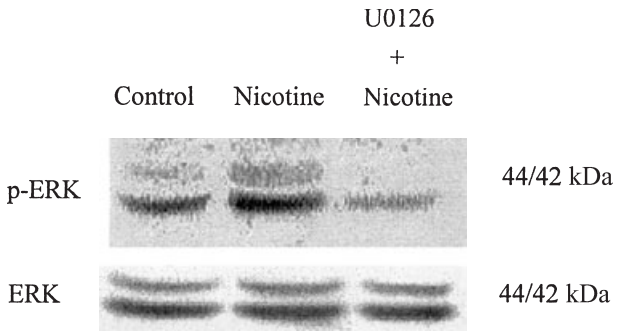

Fig. 4. Treatment of U2OS cells with 23 μM U0126 decreased nicotine-mediated phosphorylation of extracellular signal-regulated kinase (ERK). However, U0126 did not alter the total amounts of ERK.

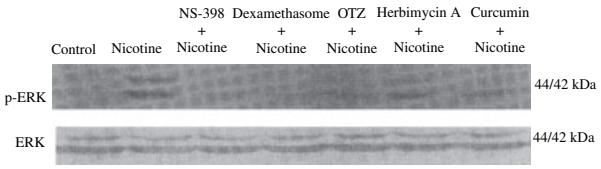

Fig. 5. Effects of various pharmacological agents on nicotine-induced phosphorylated extracellular signal-regulated kinase (p-ERK) expression in U2OS cells. Cells were co-cultured with NS-398, dexamethasone, 2-oxothiazolidine-4-carboxylic acid (OTZ), herbimycin A, and curcumin in the presence of 5 mM nicotine for further 4 h. All of the pharmacological agents were found to inhibit p-ERK activity ($p < 0.05$).

mechanisms on nicotine-induced p-ERK expression. These pharmacological agents were found to inhibit the nicotine-induced p-ERK expression ($p < 0.05$) (Fig. 5).

Discussion

Cigarette smoking is a major risk factor of periodontitis, although the molecular pathogenic mechanism remains unestablished. Molecular toxicological investigations have demonstrated that nicotine can induce c-*fos* (11) and COX-2 (12) gene expression in the fibroblasts derived from periodontium. However, there have been no previous studies of nicotine-induced transduction signaling proteins associated with cigarette smoking related-periodontal diseases.

To further investigate the mechanism of nicotine-induced signaling proteins, western blot analysis of the phosphorylated proteins was performed. Phosphorylation of ERK was increased, whereas no increase was observed in p38 MAPK and JNK. Our results were in agreement with Mai *et al.* (21) who reported that nicotine treatment resulted in the significant induction of p-ERK and had no effect on either p38 MAPK or JNK, respectively, in NCI-H82 cells. Moreover, a recent study has shown that exposure to Benzo[*a*]pyrene, a polycyclic aromatic hydrocarbon present in tobacco smoke, increases p-ERK signaling in human arterial vascular smooth muscle cells (22). Taken together, these results suggest that tobacco products-induced transduction signaling proteins is via the ERK pathway.

Increasing evidences suggest that oxidative stress is involved in many chronic inflammatory diseases associated with cigarette smoking (23, 24). Recently, nicotine was found to significantly deplete intracellular glutathione (GSH) in human periodontal ligament fibroblasts (11). To determine whether GSH levels modulated p-ERK expression in U2OS cells by nicotine, confluent monolayers were treated with OTZ. In this study, OTZ was found to inhibit nicotine-stimulated p-ERK expression. This indicates that nicotine-stimulated p-ERK expression may be partially related to the GSH levels.

COX-2 is an inducible enzyme believed to be responsible for prostaglandin synthesis at sites of inflammation. Recently, from our *in vitro* experiments, we showed that nicotine is capable of stimulating COX-2 mRNA and protein expression in human gingival fibroblasts (12). In this

study, the COX-2 inhibitor NS-398 was found to inhibit nicotine-stimulated p-ERK expression. This indicates that the signal transduction pathway COX-2 may be involved in the nicotine-stimulated p-ERK expression.

In this study, we found that dexamethasone can inhibit nicotine-stimulated p-ERK expression in U2OS cells. It is known that dexamethasone is a potent inhibitor of NF-κB activation (25). Recently, Anto *et al*. (26) found that cigarette smoke condensate activates NF-κB in human histiocytic lymphoma U937 cells. Thus, nicotine-stimulated p-ERK in U2OS cells may be partially mediated through the NF-κB pathway.

Curcumin, a dietary pigment responsible for the yellow color of curry, was also demonstrated to reduce nicotine-stimulated p-ERK expression in U2OS cells. It is well known that curcumin is an AP-1 inhibitor (27). Consistently, our recent study has shown that nicotine can induce c-*fos* gene expression in human periodontal ligament fibroblasts (11). Taken together, these results suggest that the AP-1 signal transduction pathway may be involved in the nicotine-stimulated p-ERK expression.

Tyrosine kinase inhibitors have been shown to be involved in the signal transduction pathways of MAPKs in bovine adrenomedullary chromaffin cells (28). In the present study, we found that herbimycin A can inhibit nicotine-stimulated p-ERK expression in U2OS cells. This indicates that nicotine-stimulated p-ERK in U2OS cells may be partially mediated through the tyrosine kinase pathway.

As far as we know, this is the first attempt to evaluate the role of MAPKs expression in U2OS cells stimulated with nicotine. Data from our *in vitro* experiments showed that strong phosphorylation of ERK was detected. This suggests that one of the pathogenic mechanisms of cigarette smoking-related periodontal disease *in vivo* may be the stimulation of p-ERK activity by resident cells in smokers. In the present study, p-ERK activity was inhibited by NS-398, herbimycin A, OTZ, curcumin, and dexamethasone. Therefore, studying the signal transduction pathway involved in ERK expression may prove versatile. However, more detailed studies should be undertaken to clarify the agents that can regulate p-ERK activity *in vitro* and *in vivo*. Further studies will be of importance to address the contribution of various tobacco products in the cigarette smoking-related periodontal disease.

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