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Nicotine inhibits human gingival fibroblast migration via modulation of Rac signalling pathways

Yiyu Fang and Kathy K. H. Svoboda

Biomedical Sciences Department, Texas A&M University System, Health Science Center, Baylor College of Dentistry, Dallas, TX, USA

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Abstract

Aim: Cigarette smoking is a risk factor in the development of periodontal diseases. In addition, a delayed healing process has been shown in smokers compared with non-smokers after periodontal treatment. Cell migration is a key process of wound healing and it is highly regulated by a variety of signalling pathways. The small G protein, Rac, is necessary for cell migration. Our aim was to determine if nicotine disrupted Rac and its downstream signalling proteins, p21-activated kinase 1/2 (PAK1/2), and p44/42 mitogen-activated protein kinase (MAPK) (extracellular regulated kinase 1/2).

Material and Methods: Primary human fibroblasts from healthy gingival tissues were cultured and grown to confluence. Cells were serum starved for 24 h, and then treated with nicotine (0 or 0.5 μM) prior to in vitro wounding. Cell migration was analysed in live cell assays following in vitro wounds. Rac activity, phosphorylation levels of PAK1/2, and p44/42 MAPK were assessed in cultures treated with or without nicotine after multiple wounds.

Results: Nicotine decreased cell migration rates by 50% compared with controls. In addition, nicotine altered the activation patterns of Rac and PAK 1/2 and up-regulated p44/42 MAPK.

Conclusion: Decreased cell migration in periodontal wounds exposed to nicotine may be mediated through the Rac and PAK1/2 signalling pathways.

Key words: cell migration; erk1/2; human gingival fibroblasts; MAP kinase; nicotine; PAK1/2; Rac

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It has been well documented that cell migration is a key aspect of many biological processes, including wound healing, tumour cell invasion, and embryonic development. During the wound-healing process, epithelial cells at wound edges start to migrate and proliferate to cover the denuded area. This cell migration is necessary for re-epithelialization (O'Toole 2001). At the same time, fibroblasts migrate into the wound area, proliferate, and participate in the formation of granulation tissue (Hakkinen et al. 2000).

Rho family G proteins, which include Rho, Rac1, and Cdc42, interact with each other and play an important role in regulating cell migration (Evers et al. 2000). Many investigations have demonstrated that Rho family G proteins cycle between an active guanosine triphosphate (GTP) bound form and an inactive guanosine diphosphate (GDP) bound form (Van Aelst & D'Souza-Schorey 1997, Ridley 2001). Once activated, these proteins signal through a variety of effectors, thereby regulating diverse cellular events (Etienne-Manneville & Hall 2002). We concentrated the current investigation on Rac and one of its downstream proteins, p21-activated kinases (PAKs). These proteins regulate actin re-organization and have a role in cell migration (Aspenstrom 1999, Jaffer & Chernoff 2002). Once activated, PAKs signal through other proteins including myosin light chain kinase, myosin I heavy chain, myosin II heavy chain, and desmin to regulate actin cytoskeleton dynamics and cell motility (Bokoch 2003).

In addition, other pathways, including mitogen-activated protein kinases (MAPKs) and integrins regulate actin cytoskeleton and cell motility. The activated p44/42 MAPK proteins are also named the extracellular regulated kinase (erk 1 and 2). These proteins promote cell mobility; whereas, inhibiting erk

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activation decreases cell migration (Crean et al. 2002, Pintucci et al. 2002).

Material and Methods

Tissue culture

Human gingival tissues from five healthy non-smokers (patients were numbered 1–5) having therapeutic crown lengthening surgeries were collected with Institutional Review Board-approved protocols and informed consent. Epithelial layers were removed before tissues were minced. Gingival tissues were cultured in Dulbecco's modified Eagle medium with 10% foetal bovine serum (FBS) and 1% antimycotic/antibiotic (GIBCO™ Invitrogen Corporation, Carlsbad, CA, USA). Human gingival fibroblasts (HGFs) grew from the tissues after 1 week, and the cells from passages 3 to 6 were used for experiments.

In vitro wounding

To decrease the effects of growth factors in serum, confluent HGFs (patients 4 and 5) were serum starved for 24 h, and then were treated with or without 0.5 μM (0.112 μg/ml) nicotine hemisulphate salt (Sigma, Saint Louis, MO, USA) for 2 h before in vitro wounding using a pipette tip (Coon et al. 2002). Images of the same wound areas (11 consecutive areas/treatment group) were obtained at various times using a Nikon inverted microscope equipped with a charge-coupled device digital camera (Meridian Instrument Company, Kent, WA, USA). The cells were maintained in a tissue culture incubator between image acquisitions. The cells were at room temperature (rt) for 15–20 min. for photography. HGFs (patients 1 and 2) were immunolabelled with a specific Golgi (GM130) and nucleus (propidium iodide) markers to determine cell polarity at the wound margin.

Multiple wounds were made using a plastic comb and the cells were washed quickly to remove the floating detached cells. Western blot analysis and Rac activation assays were completed on wounded monolayers that were cultured in the serum-free cell culture medium in the presence or absence of 0.5 μM nicotine hemisulphate salt for different times.

Western blot

Western blots were performed using standard protocols. HGF cells were grown to confluence in 60 mm culture dishes as described above (Fig. 2b – patients 3 and 4; Fig. 3a – patients 1, 2, and 3; Fig. 3b – patient 4). At various times, the cells were homogenized in lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.3 M NaCl, 2% octylphenyl-polyethylene glycol) with 1 × protease inhibitor cocktail, 1 × phosphatase inhibitor cocktails I and II (Sigma). The protein levels were quantified using Folin Lowry assays and stored at − 20°C. Protein samples (10 μg/lane) were separated on 7.5–15% acrylamide gels (Bio-Rad, Hercules, CA, USA) under denaturing conditions and transferred to Immobilon-P membranes (Millipore Corporation, Billerica, MA, USA). Blots were re-hydrated and blocked with 5% milk (Bio-Rad) or 5% bovine serum albumin (BSA) in Tris-buffered saline/0.1% Tween-20 (TBST) for 1 h at rt, and then incubated with primary antibodies in 5% milk or 5% BSA in TBST overnight at 4°C. Antibodies and dilutions were Rac1 rabbit polyclonal antibody (1:500) (Cytoskeleton, Denver, CO, USA), phospho-PAK1 (Ser199/204)/PAK2 (Ser 192/197) rabbit polyclonal antibody (1:1000) (Cell Signaling Technology, Beverly, MA, USA), and Phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal antibody (1:1000) (Cell Signaling Technology). Membranes were washed and incubated with 1:2500 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc. CA, USA) for 1 h at rt, and then detected using the femto LUCENT® (Geno Technology Inc., Saint Louis, MO, USA). Western blots were repeated at least three times; however, statistical analysis was not completed comparing results from different Western blots because this is not a quantitative technique.

Indirect immunocytochemistry

Indirect immunocytochemistry was performed with standard procedures (Nobes & Hall 1999). Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 15 min. at various times after wounding. Then they were rinsed with PBS, permeabilized with 0.2% Triton X-100 for 2 min., washed with PBS, and blocked with 3% BSA (Sigma) in PBS for 15 min. The Golgi apparatus was labelled with GM130 mouse monoclonal antibody (BD Biosciences, San Diego, CA, USA; 1:50 dilution in 3% BSA/PBS) overnight at 4°C. The primary antibody was detected with Alexa Fluor® 488 Goat-anti-Mouse (Molecular Probes, Eugene, OR, USA; 1:100) for 2 h at rt. Nuclei were labelled with 1 μg/ml propidium iodide (Molecular Probes) for 5 min. at rt and washed with PBS. The double-labelled cells were then analysed with confocal microscopy (Leica TCS-SP2, Leica Microsystems, Heidelberg, Germany).

Rac activation assay

Patient 4's HGFs were plated onto 60 mm tissue culture dishes and grown to confluence as described for Western blots. Cells were harvested at various time intervals post-wounding. The Rac activation assay (Cytoskeleton) was performed as previously described (Waterman-Storer et al. 1999). An equal amount of cell lysate was prepared according to the manufacturer's protocol and incubated with PAK-protein-binding domain (PAK-PBD) beads at 4°C on a rotator for 1 h. The PAK-PBD beads were pelleted by centrifugation and the supernatant was removed. To ensure that equal amounts of protein were in each sample, supernatant protein concentration was determined by Folin Lowry assays. The beads were washed in lysis buffer and wash buffer (25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl) as per the manufacturer's protocol. The beads were re-suspended in 10 μl of sodium dodecyl sulphate (SDS) sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). Each sample, including beads, was separated on a 12% SDS-PAGE gel well (Bio-Rad) and then transferred to Immobilon-P membrane for Western blot analysis. Rac1 was detected with the rabbit polyclonal antibody (1:500) as described in Western blots. Three controls provided in the assay kit (Cytoskeleton) were used in each experiment including positive cellular protein control (GTP-Rac), negative cellular protein control (GDP-Rac), and Rac1-His protein control. These controls ensure that the data obtained from the pull-down assay are valid.

Statistics

Statistical significance was tested using analysis of variance (ANOVA) for unpaired comparisons between groups. $p < 0.05$ indicated a significant statistical difference for the wound area calculations and the polarized cell analysis. Results were reported as mean ± standard error.

Results

Nicotine inhibited HGF migration in a time-dependent manner

Live cell assays were used to verify the effects of nicotine on cell migration in a serum-free environment. Confluent HGFs were serum starved for 24 h, and then treated with or without 0.5 μM (0.112 μg/ml) nicotine for 2 h before scrape wounding. The cultures were monitored for 48 h. The scrape-wounding technique produced large variable areas denuded of HGF cells. The goal of these experiments was to observe the same areas over 2 days. Therefore, 11 consecutive wound areas in each group were observed at 6, 12, 24, 36, and 48 h (Figs 1a–d). The wound areas were analysed for each time point using a Metamorph stereological program (Fig. 1e). In the serum-free conditions, neither the control nor nicotine-treated cells closed the in vitro wound. The mean open areas at the beginning of the experiment were similar 0.100 mm^2 (controls) and 0.117 mm^2 (nicotine treated). These values were used to determine the change in wound area as a percentage of the original area (Fig. 1e controls, black diamonds; nicotine treated, open squares). The control cells had higher variability but closed the wound faster than nicotine-treated cells. The control cells covered nearly 19% of the wound by 48 h, while the nicotine-treated cells decreased the wound by only

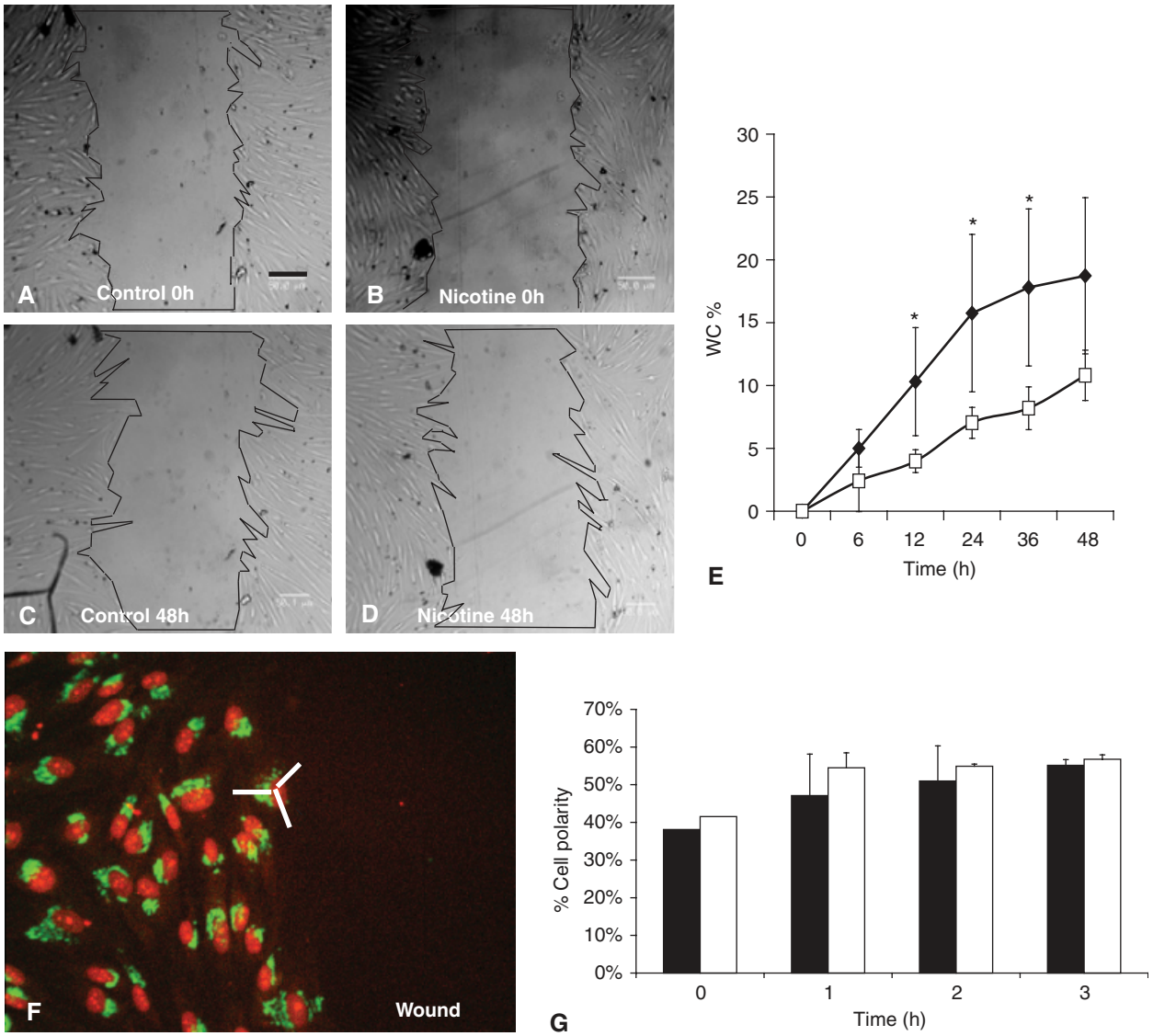

Fig. 1. (A–E) Analysis of in vitro wounds using live cell imaging: representative images of live cells immediately after wounding (A, B) or 48 h later (C, D). Scale bar: 50 μm. Human gingival fibroblasts (HGFs) that were pre-treated with 0.5 μM (0.112 μg/ml) nicotine (B, D) for 2 h before in vitro wounding and compared with controls that were not exposed to nicotine (A, C). Wound areas were tracked by using an outline program in Metamorph program that measured the area of the in vitro wound. Eleven consecutive microscopic fields were analysed and averaged. The wound closure percentage was calculated by dividing the wound area (6, 12, 24, 36, and 48 h) by the original area (E). There was no significant difference between groups at 0 and 6 h or after 36 h. However, there was a statistically significant difference ($p < 0.05$) between groups at 12, 24, and 36 h after wounding (*). (F–G) Analysis of cell polarity during cell migration. HGFs were pre-treated with 0.5 μM nicotine for 2 h before in vitro wounding. The Golgi apparatus were immunohistochemically stained (green) and nuclei were labelled with propidium iodide (red). The distribution of the Golgi apparatus immediately (0) after wounding (F) was compared 1, 2, and 3 h after wounding (G). Approximately 39% of the cells had polarity as measured by Golgi location in the cytoplasm facing the wound immediately (0). The percentages of cells having polarity increased in both control (black bars) and nicotine-treated (open bars) cells. However, there was no statistically significant difference between groups.

10.8%. The nicotine-treated cells were migrating at half the rate of control cells at all times examined in this study. There was a statistically significant difference in wound closure rates at 12, 24, and 36 h (Fig. 1e, *) using ANOVA analysis for unpaired groups ($p < 0.05$). As all cultures in the experiments were treated equally at rt for 15–20 min. before each recording session, future experiments would benefit from an environmental chamber.

Nicotine did not alter cell polarity

Disrupting cell polarity has been shown to inhibit fibroblast migration (Bershadsky & Futerman 1994). Therefore, we wanted to determine if nicotine impaired cell polarity at the wound margin. The Golgi apparatus location in comparison with the nucleus was used to determine cellular polarity. HGFs were pre-treated with 0.5 μM nicotine for 2 h before in vitro wounding. The Golgi apparatus were immunohistochemically stained (GM130) and nuclei were labelled with propidium iodide. The distribution of the Golgi apparatus was compared between nicotine-treated and control cultures immediately (0), 1, 2, and 3 h after HGFs were wounded (Fig. 1f). Cells along the wound edge were divided into three 120° sections using an overlay vector (Fig. 1f). The vector centre was on the nucleus of each cell (Fig. 1f). Cells with the Golgi apparatus within the 120° section facing the wound were defined as having polarity, whereas, if the Golgi was in either lateral area, the cell was considered non-polar towards the wound margin (Nobes & Hall 1999).

Immediately after wounding (0 h), the percentage of wound edge cells having polarity was 38.10% in controls (Fig. 1g, black bars) and 41.54% in nicotine-treated (Fig. 1g, open bars) cultures. The percentage of polarized cells increased in both groups 1, 2, and 3 h after wounding. However, compared with control groups, nicotine treatment did not change the percentage of the cells with polarity (Fig. 1g). We concluded that nicotine did not change the ability of the cells to sense the migration direction needed to close the wound under these experimental conditions.

Nicotine changed Rac and PAK1/2 activities during cell migration

Many different signal transduction proteins contribute to cell migration. The small Rho family G protein, Rac, increases lamellipodia during cell migration (Nobes & Hall 1999, Evers et al. 2000, Ridley 2001). We used pull-down assays that only detect the activated GTP-bound Rac to determine if it becomes activated in the in vitro wound model and if nicotine changes Rac signalling. The HGF cells were cultured to confluence, serum starved, treated with nicotine, and then wounded. Cell lysates were collected from 5 min. to 1 h. The Rac pull-down assay uses a protein domain that only binds to GTP-Rac and does not bind to GDP-Rac. The controls for the assay worked perfectly as the control GTP-Rac and His-tagged Rac had strong signals, while the GDP-Rac did not have a signal on the Western blots (Fig. 2a, lanes marked GTP, H-Rac, and GDP). In the control-wounded cultures, Rac was activated at 5 min. after wounding, but oscillated between the GTP and GDP states. Rac was active at 5, 20, and 40 min. but inactive at 10, 30, and 60 min. Furthermore, the Rac signal at 5 and 40 min. was nearly as strong as the GTP control. In contrast, the nicotine-treated group had less activated Rac at all time points, but some activated Rac was detectible at 10 and 30 min. (Fig. 2a).

Active Rac binds to PAK producing a conformational change and autophosphorylation on specific sites; thus, the activation of PAK is another indication of Rac activity (Zenke et al. 1999). As an alternative approach, we determined if nicotine had any effect on PAK1/2. PAK1/2 activity was determined by a specific phospho-PAK1/2 antibody. Interestingly, in our culture system, control cells had very low levels of activated PAK1/2 after wounding (Figs 2b and c, black bars). In contrast, nicotine up-regulated PAK1/2 activity in the first 3 min. after wounding and continued through 5, 10, and 20 min. time points (Figs 2b and c, open bars). We concluded from these results that other signalling processes may be increasing PAK activity and it was not related to Rac activity under these experimental conditions.

Nicotine up-regulated MAPK pathways

Many other signalling molecules can also be involved in cell migration that may or may not be downstream of Rac. One of these proteins is a MAPK family member, MEK (mitogen-activated kinase kinase) that has a downstream marker, erk 1/2, which is detected with a phosphor-specific antibody (P-p44/42). This pathway is involved in cell migration through control of the actin cytoskeleton; we investigated if nicotine had a direct effect on MAPK activity. In contrast to the previous experiments that recorded the activity of target proteins, MEK function can be determined by using a pharmacological agent that directly and specifically inhibits its activity. HGFs were pre-treated with 0 or 100 μM MEK-1 inhibitor PD98059 for 2 h. Either 0 or 0.5 μM of nicotine was added to the culture medium and cultured for 0, 30, or 60 min. in cells from three patients (Fig. 3a, patients 1, 2, or 3). The experiments demonstrated that erk 1/2 (Fig. 3a, P-p44/42) was activated by nicotine in a time-dependent manner. In addition, PD98059 inhibited this nicotine-induced activation (Fig. 3a).

In order to study if nicotine regulates erk 1/2 activity during cell migration, in vitro wound models were used. HGFs were pre-incubated with or without nicotine for 2 h before multiple wounding. In both control and nicotine-treated cells, activated erk 1/2 (P-p44/42) levels were detectible, but were low (Fig. 3b). The control cultures responded to multiple wounds by increasing phosphorylation of erk 1/2 (P-p44/42) with a peak at 3–5 min. and returned to baseline levels by 15 min. (Fig. 3b, black bars). In contrast, in the nicotine-treated cultures, erk 1/2 was activated by 30 s, reached a peak at 3 min. followed by a plateau from 5 to 15 min. before returning to baseline levels at 40 min. (Fig. 3b, open bars). Interestingly, in nicotine-treated groups, erk 1/2 activity was always more intense than in control groups at the same time point after wounding (Fig. 3b).

Discussion

Cell migration is a critical event in gingival wound healing (Hakkinen et al. 2000). In this investigation, we examined some of the possible signal transduction pathways necessary for fibroblast migration to confirm that nicotine has a detrimental effect on this critical cellular activity during wound healing. The experiments were performed using an in vitro wound-healing model under highly controlled conditions that excluded or decreased the influence of growth factors from foetal calf serum. In addition, we used a live cell assay to measure wound closure over a defined time and found that nicotine decreased wound closure rates significantly from 12 to 36 h. However,

the differences between groups were small and by 48 h insignificant. Further experiments utilizing an environmental chamber and constant monitoring may reveal more details concerning the cells migration behaviour. We also examined the activation of specific signalling proteins that may contribute to cell–matrix interactions or actin cytoskeleton rearrangements. Our results demonstrated that nicotine decreased activated Rac, but increased activated PAK1/2 and erk 1/2 (P-p44/42 MAPK). Furthermore, we found that nicotine had no effect on cell polarity at the wound edge. Topics that will be discussed include the following: nicotine concentration, comparison of these data with other experiments examining nicotine effects on cell migration, cell polarity during migration, Rac, PAK, and erk results, and possible signalling pathways (Fig. 4).

The concentration of nicotine (0.5 μM or 0.112 μg/ml) used in this study was within the normal serum concentration of smokers. Concentration of nicotine in human serum varies depending on the levels of smoking (Lawson et al. 1998). We found that fibroblasts treated with 0.5 μM nicotine moved at approximately half the rate of control cells. A similar conclusion was obtained using gastric epithelial cells in live cell assays and serum-free conditions with much higher doses of nicotine, 200 μg/ml (Shin et al. 2002). A recent study using cigarette smoke reported that dermal fibroblasts and chick embryo fibroblasts decrease migration with increased cell adhesion (Wong & Martins-Green 2004). However, the effect of nicotine on cell migration has been controversial, because some studies

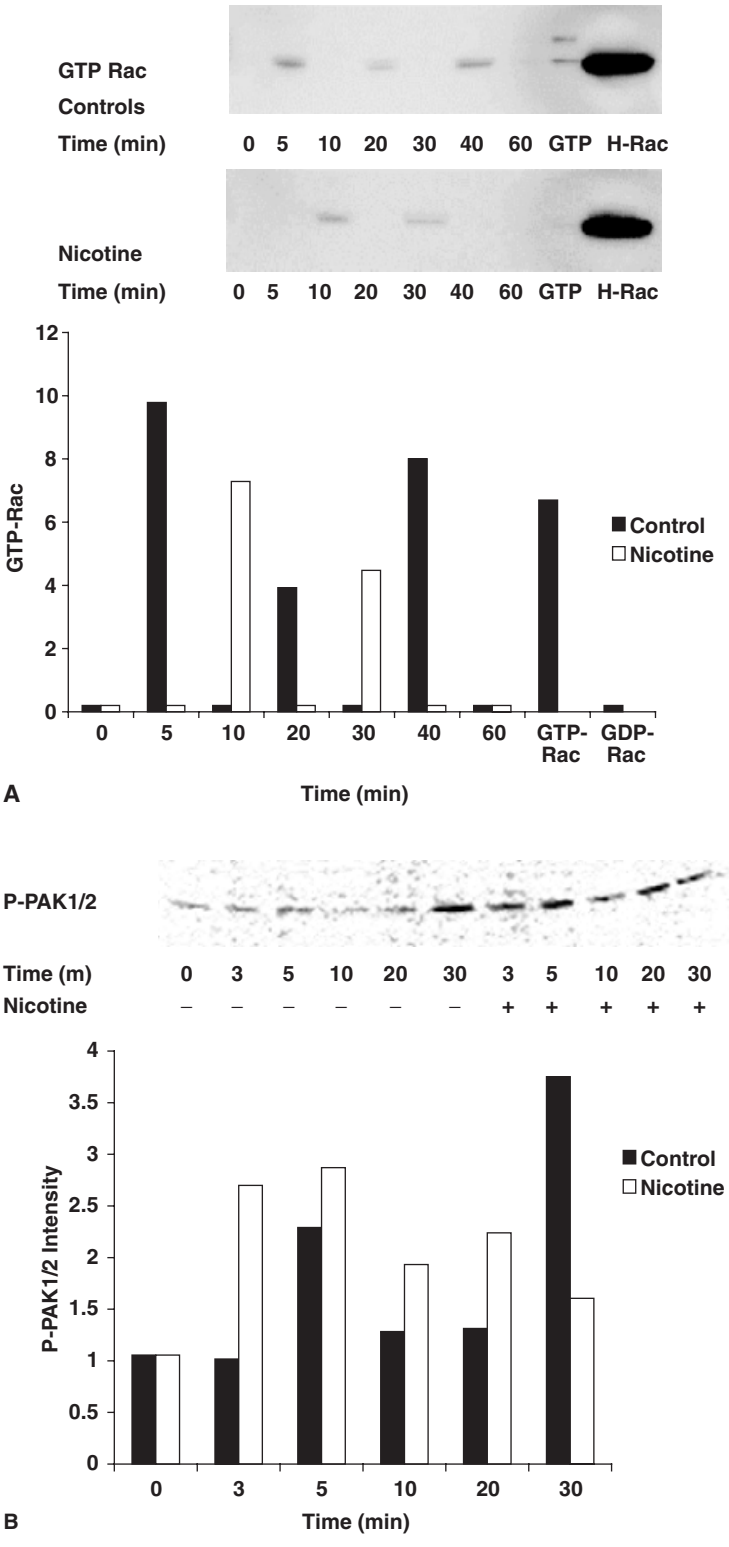

Fig. 2. Nicotine changed Rac (A) and p21-activated kinase 1/2 (PAK1/2) (B) activities during cell migration. Cells were treated with or without 0.5 μM of nicotine immediately prior to in vitro wounding. Cell lysates were collected at 0, 5, 10, 20, 30, 40, and 60 min. after wounding and activated Rac1 (guanosine triphosphate (GTP) bound, GTP-Rac) was precipitated in a pull-down assay (A). Experimental controls included GTP-Rac (top gel, GTP), guanosine diphosphate (GDP)-Rac (bottom gel, GDP), and His-tagged Rac (H-Rac). All controls worked as expected with GTP-Rac and H-Rac having positive bands, while GDP-Rac signal was not detectible. GTP-Rac oscillated and was pulled down in the assay at 5, 20, and 40 min. in control HGF cultures. In comparison, GTP-Rac in nicotine-treated cells was decreased in intensity and delayed. Small amounts of GTP-Rac were pulled down at 10 and 30 min. after wounding. All experimental samples started with the same amount of protein and the assay was repeated two times. (B). Phospho-PAK1/2 (P-PAK1/2) was analysed in Western blots of cell lysates from untreated control cells and nicotine-(0.5 μM)-treated cells after in vitro wounds at 3, 5, 10, 20, and 30 min. Compared with untreated control cells (0 time point), nicotine up-regulated PAK1/2 activity in the first 3 min. after wounding. Densitometry of this Western blot (B, graph) of phospho-PAK1/2 demonstrated that control cells (black bars) increased PAK1/2 at 5 and 30 min., whereas nicotine (open bars) up-regulated PAK1/2 from 3 to 20 min. All lanes were loaded with the same amount of total protein and the experiment was repeated three times.

found that nicotine stimulated lateral migration of keratinocytes (Grando et al. 1995), while others showed that nicotine had no direct effect on rabbit gastric cell migration (Sato et al. 1994). The difference may be because of different nicotine exposure times, source of nicotine, presence of foetal calf serum or other growth factors, cell types, direction of migration, and/or other assay conditions.

Directed cell migration requires dynamic polarization of cells. In migrating fibroblasts during wound healing, Golgi apparatuses are re-orientated towards the migration direction (Fukata et al. 2003). Disruption of the Golgi apparatus re-orientation inhibited mouse 3T3 cell migration (Bershadsky & Futerman 1994). Our results demonstrated that cell polarity was not affected by nicotine in the first 3 h. Re-organization of the microtubular network is another characteristic of polarized cells (Fukata et al. 2003), and disruption of microtubules inhibits wound closure (Nobes & Hall 1999). However, an increase in microtubules that appeared less organized was reported in dermal fibroblast cells after exposure to cigarette smoke (Wong & Martins-Green 2004). In contrast, these investigators reported an increase in F-actin and vinculin, a focal adhesion protein. They hypothesized that the nicotine-treated cells were more adherent to the substrate and that may contribute to the slower migration rate (Wong & Martins-Green 2004). Recently, it was shown that integrin expression ($\beta 1$ *versus* $\beta 3$) may also change migration patterns through the small GTPases (Rho *versus* Rac) (Danen et al. 2005).

Several theories have been suggested to explain nicotine-inhibited cell migration including increased cell adhesion

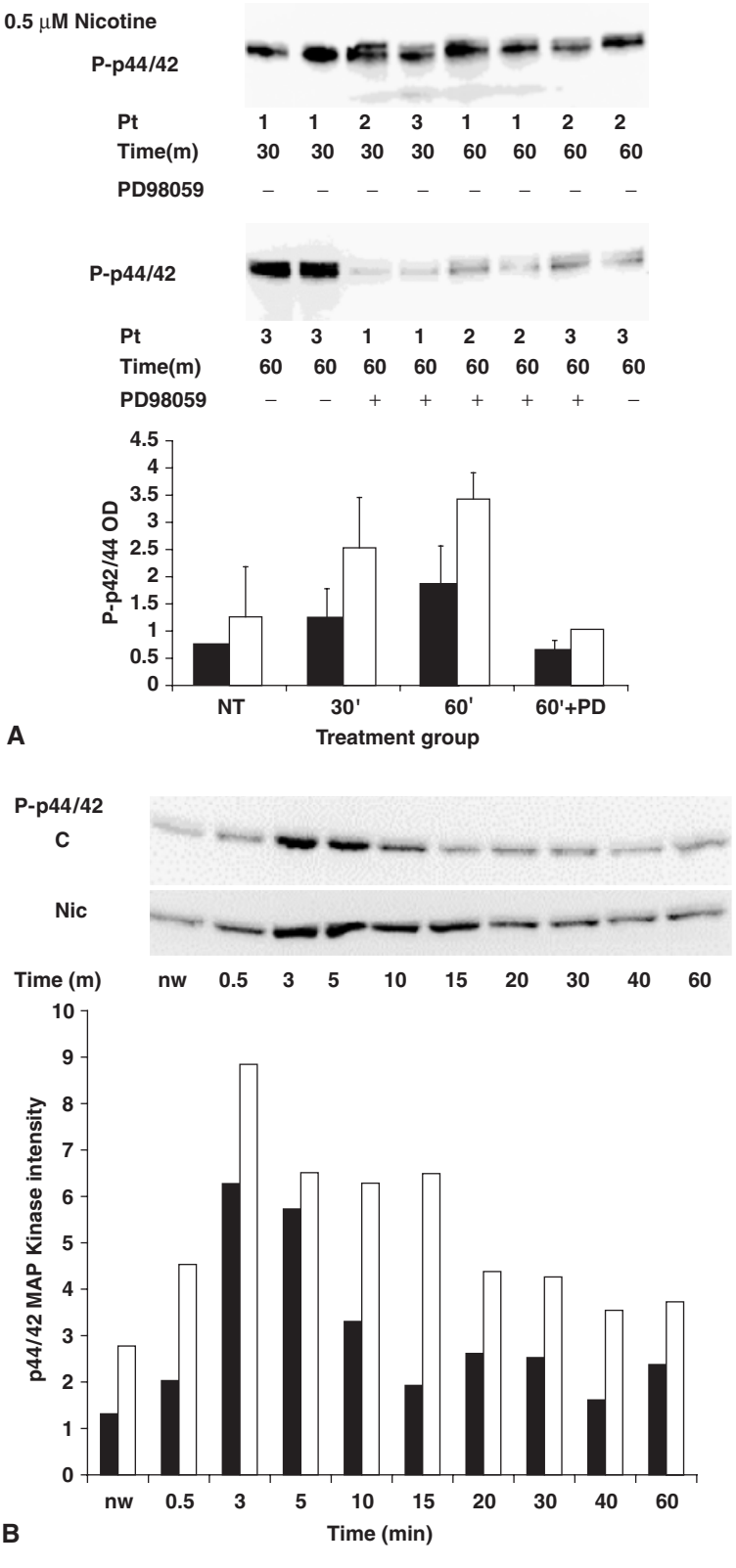

Fig. 3. Nicotine up-regulated mitogen-activated protein kinase (MAPK) activity before and during cell migration. (A) Human gingival fibroblasts (HGFs) from three non-smokers (patients 1, 2, and 3) were pre-treated with or without the mitogen-activated kinase kinase (MEK) inhibitor, PD 98059, for 2 h before treatment of nicotine (0 or 0.5 μM) for 30 or 60 min. (Time (m)). Western blot analysis for phosphorylated extracellular regulated kinase (erk) 1/2 (P-p44/42) was completed. Nicotine-induced phosphorylation of p44 (open bars) and 42 (black bars) erk 1/2 increased approximately twofold after 30 min. and 2.7-fold after 60 min. of treatment (A, graph) that was blocked in the presence of the MEK inhibitor, PD98059 (100 μM). (B) Cells were pre-treated with or without 0.5 μM nicotine for 2 h prior to in vitro wounding. Cell lysates were collected (0.5, 3, 5, 10, 15, 20, 30, 40, and 60 min.) and phosphorylation levels of erk 1/2 (P- p44/42 MAPK) were analysed by Western blots. All cells responded to in vitro wounding by increasing phosphorylation of p44/42 at 3 and 5 min. (B) Western blots and graph. By 10 min. the control cells had decreased phosphorylation (black bars), while the nicotine-treated cells (open bars) up-regulated erk 1/2 activity from 3 to 15 min., and then decreased to twofold higher than controls from 20 to 60 min.

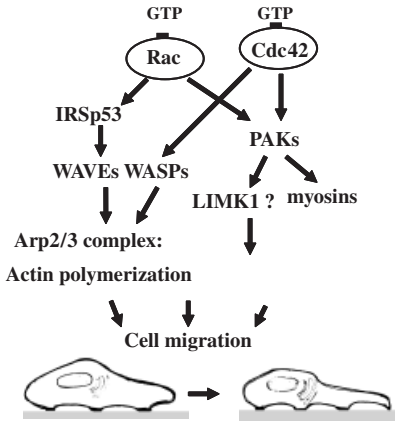

Fig. 4. Schematic drawing of the signal transduction pathways involved in human gingival fibroblast (HGF) migration including the possible effects of nicotine on these pathways. The small guanosine triphosphate (GTP)ase, Rac, becomes activated when a GTP is bound to the protein. The GTP-Rac activates p21-activated kinases (PAK 1/2). PAK can also be activated by pathways independent of Rac (other kinases). Activated PAK1/2 signals through other proteins including myosin light chain kinase (MLCK), myosin I heavy chain (MHC), myosin II heavy chain (MIIHC), and desmin to regulate actin re-organization and cell migration. Rac can also activate the Wiskott–Aldrich syndrome protein (WASP) and vasodilator-stimulated phosphoprotein (VASP) family. Signals from these proteins integrate into the Arp2/3 complex that increases actin polymerization. The cell–matrix interactions through integrins are partially regulated by mitogen-activated protein kinases (MAPKs) to regulate actin cytoskeleton and cell adhesion and motility. The activated p44/42 MAPK proteins are also named the extracellular-regulated kinase (erk 1 and 2). The current data showed that nicotine changed Rac activation patterns but increased PAK1/2 and erk1/2. We hypothesize that the increased erk1/2 activation may increase cell adhesion and decrease cell migration. Nicotine also appears to increase PAK1/2 activation through a Rac-independent pathway that has not been identified.

(Wong & Martins-Green 2004), modulating calcium signalling (Zia et al. 2000) and potassium ion channel activity (Shin et al. 2002). However, very few studies have investigated the effect of nicotine on the Rho family G proteins. We hypothesized that nicotine inhibits cell migration by affecting Rac signalling. Our results suggested that nicotine decreased and delayed the activation pattern of Rac. In our investigations, we concentrated on examining the active form of several proteins, as the cellular pool of inactivated signalling proteins is usually constant. The GTP-Rac assay depends on protein–protein binding; therefore, controls are extremely important to interpret the results. This type of pull-down assay is similar to immunoprecipitation, except that the target protein (Rac) only binds to the bait (PAK-PBD) if it is activated. Therefore, the assay is very sensitive and records the amount of GTP-Rac in the samples.

Once it was established that GTP-Rac was influenced by nicotine, we determined if nicotine delayed the activation of PAK1/2, downstream effectors of Rac (Aspenstrom 1999). Interestingly, we found that nicotine up-regulated PAK1/2 activation even 3 min. after wounding. Even though PAK1/2 are normally accepted as one of the effectors of Rac and Cdc42, there are data that PAK could also be activated by pathways independent of Rac or Cdc42. For example, PAK activation occurred with mutated molecules that prevented binding of Rac or Cdc42 (Bokoch et al. 1998). It is possible that nicotine up-regulated PAK1/2 activity through signalling molecules other than Rac/Cdc42. In addition, research using human microvascular endothelial cells found that either unregulated increases or decreases in PAK activity inhibited cell motility (Kiosses et al. 1999). Recent investigations have shown that moderate cigarette smoke increased the expression of cell survival proteins IL8, p53, and p21 while decreasing cell migration in dermal fibroblasts (Wong & Martins-Green 2004). Therefore, nicotine affected the proper regulation of PAK, which was required for HGF migration. Besides PAK1/2, other effectors such as Wiskott–Aldrich syndrome protein and vasodilator-stimulated phosphoprotein (VASP) family also link Rac to the actin cytoskeleton. Signals from these proteins integrate into the Arp2/3 complex, which in turn lead to actin polymerization (Fig. 4) (Ridley 2001). Future studies are needed to analyse if nicotine affects the activities of these signalling molecules.

Several studies have suggested that erk 1/2 activity was up-regulated by nicotine and signals were transduced through the nicotinic receptor (Dajas-Bailador et al. 2002, Tomizawa & Casida 2002). It was also well documented that erk 1/2 activity was important for cell migration (Crean et al. 2002) and was up-regulated in cell–matrix interactions (Chu et al. 2000). Therefore, we investigated if nicotine has an effect on erk 1/2 activity. Our results demonstrated that erk 1/2 was up-regulated by nicotine before and during cell migration. As nicotine inhibited HGF migration and increased phosphorylation of erk 1/2, the cells may become more adherent to the substrate to decrease migration rates, similar to the dermal fibroblasts (Wong & Martins-Green 2004).

We did not investigate if the inhibition of HGF migration was signalling through the nicotinic receptor. It has been well demonstrated that nicotinic receptors exist on non-neuronal cells (Sharma & Vijayaraghavan 2002). A palate study from our lab demonstrated that nicotine inhibited epithelial–mesenchymal transformation by signalling through the nicotinic receptor (Kang & Svoboda 2003). Our future studies would be designed to detect if nicotinic receptors become up-regulated after nicotine treatment, and if the inhibition of cell migration is induced through the nicotinic receptor. In addition, we would like to determine if the effect of nicotine on Rac and PAK1/2 signalling is transduced through the nicotinic receptor.

In conclusion, our study demonstrated that nicotine decreased HGF migration by 50% in serum-free conditions and decreased Rac activation while up-regulating PAK1/2 and erk 1/2. These signalling pathways may be important modulators that can be regulated to increase oral wound healing in patients that smoke.

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Address:
Kathy K. H. Svoboda
Biomedical Sciences Department
Baylor College of Dentistry
3302 Gaston Avenue
Dallas
TX 75246
USA
E-mail: ksvoboda@bcd.tamhsc.edu

Clinical Relevance

Cigarette smokers respond less favourably than non-smokers to periodontal therapy. There is a correlation with the smoking frequency and healing response as heavier smokers have decreased healing responses (Ah et al. 1994, Kaldahl et al. 1996). Many undesirable effects of smoking have been attributed to nicotine (Campanile et al. 1998) and it is well documented that nicotine inhibits wound healing (Grossi et al. 1996, James et al. 1999, Pinto et al. 2002). However, it is not understood how nicotine affects the wound-healing processes. Although it has been shown that HGF migration is decreased in the presence of nicotine, the signal transduction pathways involved have not been identified. The purpose of this project was to determine if nicotine inhibited HGF migration through the Rac/PAK or MAPK pathways.