Reversal of Second-hand Cigarette Smoke–Induced Impairment of Corneal Wound Healing by Thymosin β4 Combined with Anti-inflammatory Agents

Hongwei Yuan,1,2 Chongze Ma,1,2 Lisa Moinet,1 Noboru Sato,3 and Manuela Martins-Green1

PURPOSE. Abnormalities in corneal reepithelialization caused by second-hand cigarette smoke (CS) are less known than the effects of CS on other tissues. The effects of CS on corneal epithelial cell migration and associated signaling mechanisms were examined, to determine the mechanisms by which CS delays corneal wound healing.

METHODS. Corneal epithelial cells in two-dimensional or organ culture were exposed to sidestream whole (SSW) smoke, a major component of second-hand CS. Thymosin beta 4 (Tβ4), a molecule thought to promote wound healing in the cornea, and it partially reversed the inhibition of corneal healing by SSW smoke. However, Tβ4 plus dexamethasone, an inhibitor of inflammation, together, reversed the effects of SSW smoke on corneal healing.

RESULTS. Cell migration, actin reorganization, and phosphorylation of focal adhesion kinase (FAK) and paxillin were all inhibited by exposure to SSW smoke, and the distribution of phospho-src in the cells was disrupted. Activation of RhoA, an important regulator of the cytoskeleton during cell migration, was also inhibited. Tβ4 stimulated corneal epithelial cell migration in the presence of SSW smoke in culture and in vivo, and it partially reversed the inhibition of corneal healing by SSW smoke. However, Tβ4 plus dexamethasone, an inhibitor of inflammation, together, reversed the effects of SSW smoke on corneal healing.

CONCLUSIONS. These findings suggest that SSW smoke exerts its effects on cell migration during corneal epithelial healing through inhibition of actin reorganization, activation of focal adhesion molecules, formation of the focal adhesion complex, and activation of Rho-GTPases. Furthermore, they strongly suggest that corneal injury induced by toxicants can be treated using anti-inflammatory agents coupled with Tβ4. (Invest Ophthalmol Vis Sci. 2010;51:2424–2435) DOI:10.1167/iovs.09-3692

It is well known that abnormal corneal reepithelialization increases the risk of pathogen invasion and further damage to the underlying stroma, resulting in cornea infection, opacity, neovascularization, and stroma ulceration.1,2 It has also been established that cigarette smoke can delay wound healing, but very little is known about its effects on corneal wounds.3–6

Our previous findings7 showed that corneal epithelial wound healing is delayed in mice that have been exposed to sidestream whole (SSW) smoke, which is the smoke that arises from the burning end of cigarettes and is the major component of second-hand cigarette smoke. We found that SSW smoke stimulates an increase in the number of neutrophils in the corneal stroma of the wounded area and that inhibition of inflammation with anti-inflammatory agents such as dexamethasone or a decrease in the number of systemic neutrophils attenuates but does not reverse cigarette smoke–induced inhibition of corneal healing. These findings indicate that mechanisms other than inflammation are involved in smoke impairment of healing. Because we also observed that the epithelial cells at the leading migrating edge do not adhere to the underlying stroma, a key process for epithelial cell migration, we hypothesized that SSW smoke causes a delay in corneal wound healing by also decreasing epithelial cell adhesion and migration.

During corneal epithelial cell migration, the epithelial cells around the wound’s edge migrate to cover the wound and restore normal cornea structure. When the corneal epithelial cells begin to migrate, cellular processes form,8 which bind to extracellular matrix (ECM) components, providing anchorage points that enable the cell to move.9,10 Cell adhesion and migration are regulated, at least in part, by the focal adhesion kinase (FAK) protein, a nonreceptor tyrosine kinase, and its downstream signaling pathways. Tyrosine phosphorylation of FAK is associated with the formation of focal complexes.9 Phosphorylation-associated FAK in turn phosphorylates focal complex-associated proteins such as paxillin.11,12 Indeed, the loss of FAK expression inhibits focal adhesion turnover, which is the continuous formation and disassembly of adhesions at the leading edge of migrating cells,13 resulting in decreased cell migration.14 Another important associated signaling molecule downstream of FAK is RhoA, a member of the family of small Rho GTPases. RhoA has been implicated in many aspects of cell migration, including the formation of actin stress fibers, focal adhesions, and membrane ruffling.15–19 For example, inhibition of RhoA activation interferes with the formation of focal adhesions and stress fibers. In contrast, microinjection of a constitutively active form of RhoA (RhoAV14) into cells induces the formation of focal adhesions and stress fibers.15,16,20 What is not known are the mechanisms by which second-hand smoke affects these molecules.

We investigated the effects of SSW smoke on corneal epithelial wound healing in vitro and in vivo. In our study, SSW smoke exerted its effects on cell migration during corneal epithelial wound healing through the inhibition of actin rearrangement, the inactivation of focal adhesion complex mole-

From the Departments of 1Cell Biology and Neuroscience and 3Biochemistry, University of California, Riverside, Riverside, California.

2Contributed equally to the work and therefore should be considered equivalent authors.

Supported by the UC Tobacco-Related Disease Research Program and the Philip Morris External Research Program (MMG). Part of this work was performed in the UCR Center for Plant Cell Biology Microscopy and Imaging Core Facility.

Submitted for publication March 12, 2009; revised September 21 and October 30, 2009; accepted November 6, 2009.

Disclosure: H. Yuan, None; C. Ma, None; L. Moinet, None; N. Sato, None; M. Martins-Green, None

Corresponding author: Manuela Martins-Green, Department of Cell Biology and Neuroscience, University of California Riverside, 900 University Avenue, BSB room 2117, Riverside, CA 92521; manuela.martins@ucr.edu.

cules, and the inactivation of Rho-GTPases. Also, Tβ4, a molecule that recently has been shown to promote corneal epithelial wound healing,21–23 reversed in part the cigarette smoke–induced delay in cornea healing by stimulating cell migration. Furthermore, when Tβ4 was applied in combination with dexamethasone in vivo, the effects of second-hand cigarette smoke on healing were reversed.

**METHODS**

**Key Reagents**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise indicated. The following primary antibodies were used: rabbit anti-β-actin (Chemicon, Temecula, CA); mouse anti-histone 2B (Cell Signaling Technologies, Beverly, MA); rabbit anti-RhoA and rabbit anti-FAK (Santa Cruz Biotechnologies, Santa Cruz, CA); mouse anti-FAK, mouse anti-paxillin, and rabbit anti-phosphotyrosine (BD Transduction Laboratories, Lexington, KY); rabbit anti-Tyr(P)31 paxillin, rabbit anti-Tyr(P)418 Src, rabbit anti-Src, and rabbit anti-Tyr(P)397 FAK (Biosource International, Camarillo, CA). The following secondary antibodies were used: goat anti-rabbit-AlexaFluor 594, chicken anti-mouse-AlexaFluor 488, goat anti-rabbit IgG-FITC, goat anti-mouse IgG-Cy3, and chicken anti-mouse-FITC (Molecular Probes, Eugene, OR). Donkey anti-rabbit IgG (Cell Signaling Technologies) or goat anti-mouse IgG (Pierce Biochemicals, Rockford, IL) conjugated to HRP was used for immunoblot analysis with extended-duration substrate (Super Signal West Dura; Pierce Biochemicals) and the ECL (GE Healthcare, Piscataway, NJ) detection system.

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933454/) Effect of SSW smoke on cell migration in HCECs. (A–D) HCECs were plated inside cloning rings and allowed to adhere for 3 hours, followed by removal of the rings. The cell edges were then marked, and the cells were treated with the SSW smoke solution or medium alone. At multiple time intervals, the migration distance was measured from the initial edge of the ring to the new cell edges. (E) Quantitative image analysis of the distance that the cells migrated at different time points in the absence or presence of the SSW smoke treatment (*P < 0.001). (F) Quantitative image analysis of the velocity of migration in the absence or presence of the SSW smoke solution. (G, H) Scratch wounds were made in confluent cultures of HCECs. After 18 hours in culture, the wounded area was largely occupied by migrating cells in the control group. In contrast, in the presence of the SSW smoke solution, very few cells migrated into the wound area. Scale bar, 100 μm.

**Figure 1.** Effect of SSW smoke on cell migration in HCECs. (A–D) HCECs were plated inside cloning rings and allowed to adhere for 3 hours, followed by removal of the rings. The cell edges were then marked, and the cells were treated with the SSW smoke solution or medium alone. At multiple time intervals, the migration distance was measured from the initial edge of the ring to the new cell edges. (E) Quantitative image analysis of the distance that the cells migrated at different time points in the absence or presence of the SSW smoke treatment (*P < 0.001). (F) Quantitative image analysis of the velocity of migration in the absence or presence of the SSW smoke solution. (G, H) Scratch wounds were made in confluent cultures of HCECs. After 18 hours in culture, the wounded area was largely occupied by migrating cells in the control group. In contrast, in the presence of the SSW smoke solution, very few cells migrated into the wound area. Scale bar, 100 μm.
Other reagents included: TpH9252 (the kind gift of Hynda K. Kleinman, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD); antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA); a protein assay kit (DC; Bio-Rad, Hercules, CA); and rhodamine-phalloidin (Molecular Probes).

Animal Experimentation

The Institutional Animal Care and Use Committee (IACUC) at the University of California Riverside approved all the animal protocols for the study. All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57Bl6 mice, 8 to 12 weeks of age, were used for the experiments.

Corneal Epithelial Injury Model

The corneal injury was performed according to published procedures.1,7 Briefly, the mice were anesthetized with injected intraperitoneally ketamine and xylazine, and a 1.5-mm diameter portion of the central cornea epithelium, including the basement membrane, was removed with a trephine and a scalpel. After the central cornea epithelium was removed, 1 drop of 0.3% ofloxacin ophthalmic solution (Ocuflox; Allergan, Irvine, CA) was topically applied to prevent infection. After awakening, the mice were injected SC with buprenorphine (Buprenex; Reckitt Benckiser Healthcare, Hull, UK), to prevent postoperative pain.

Cell Culture

Human corneal epithelial cells (HCECs) provided by Suzanne Fleiszig (University of California, Berkeley, CA) and originally developed by Araki-Sasaki et al.25 were used. The cells were cultured at 37°C with 5% CO2 in DMEM/F12 medium supplemented with HEPES (10 mM), DMSO (1:200), insulin (5 g/mL), antibiotic (1%), and fetal bovine serum (10%).

Corneal Organ Culture

Organ culture experiments were performed as described previously.26 After the central corneal epithelium was removed, the corneas were isolated and cultured in the same condition as for cell culture for 18 hours at 37°C, in a humidified atmosphere of 95% air and 5% CO2.

Preparation of SSW Smoke Solutions

SSW smoke solutions were prepared in DMEM/F12 with 1R3F research-grade cigarettes (University of Kentucky, Louisville, KY) and a method modified from a published protocol.7,27 The second-hand cigarette smoke was drawn to the medium with a puffer box built by the University of Kentucky. The pH of each solution was adjusted to 7.4 before use. To ensure that we were exposing the cells to doses of smoke similar to those found in tissues in vivo, we determined the concentration of nicotine. The level of nicotine in the SSW smoke solution was approximately 20 μg/mL per cigarette. We diluted the solution with culture cell medium to yield a final concentration of approximately 2 μg/mL, which is similar to the amount of smoke exposure of a person who spends 78 minutes in a smoke-filled room.28

Scratch Wounding of Cultured Corneal Epithelial Cells

Scratch wounding of cultured confluent corneal epithelial cells was performed as previously described.29 Briefly, confluent cultures in 35- or 100-mm culture dishes were scratched wounded with a plastic pipette tip and then cultured in cell culture medium in the presence or absence of a SSW smoke solution. After 18 hours, the cells were analyzed cytologically and biochemically. For the cell migration assays, images were obtained at different time points, to determine migration from the wound’s edge.

Cloning Ring Assay of Cultured Corneal Epithelial Cells

Corneal epithelial cells were plated at a density of 1 × 105 cells within a cloning ring. The cells were allowed to adhere for 3 hours. The edge of the ring formed by the cells was marked, and the cells were then treated with the smoke solution and allowed to migrate. At different time points after treatment, the migration distances were measured with a micrometer, from the edge of the initial ring to the final location of the migrating cells.

![Figure 2](image-url) Effect of SSW smoke on cell division in HCECs. (A) HCECs were plated and cultured for 18 hours in the absence and presence of SSW smoke treatment and then were fixed and stained with a antibody to Ki67. The cells that were positively labeled by the Ki67 antibody were counted under the microscope, the number of cells per high-power field was counted, and the results were analyzed. The difference was not statistically significant. (B) CCK-8 assay to quantify cell proliferation. HCECs were plated in a 96-well plate (5000 cells/well), cultured for 24 hours, and treated with or without SSW smoke solution in culture medium for 18 hours, followed by reaction with CCK-8 solution. Absorbance at 450 nm was measured after a 2-hour incubation. SSW did not significantly affect cell division when compared with the control.
Staining of Wholemount Cornea and Confocal Microscopy

After the removal of the central corneal epithelium, the corneas were cultured for 18 hours in the presence and absence of SSW smoke solution. They were then fixed in 4% paraformaldehyde for 2 hours, followed by washing with PBS for 1 hour and immunostaining. After the staining, the whole corneas were mounted on microscope slides, covered with mounting medium (Vectashield; Vector Laboratories), and visualized with confocal fluorescence microscopy.

Immunoblot Analysis

Eighteen hours after scratch wounding of cultured corneal epithelial cells, the cells were lysed in ice-cold TBS (25 mM Tris [pH 7.4], 0.15 mM NaCl) containing 1% Triton X-100, 5 mM EDTA, 0.5 mM pervanadate, and a protease inhibitor cocktail. The cell extracts were centrifuged at 14,000 g for 15 minutes at 4°C, and the supernatants were used for further analysis. The total protein concentration of each extract was determined with a protein assay kit (DC Protein Assay; Bio-Rad, Hercules, CA). The protein samples (30 μg per lane) were mixed with 4× sample buffer and boiled for 5 minutes before loading. They were separated by SDS-PAGE (8%–15% acrylamide) and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH). The membranes were blocked with 5% nonfat milk in TTBS (50 mM Tris [pH 7.5], 0.9% NaCl, and 0.1% Tween-20) for 1 hour at room temperature and then incubated for 2 hours at room temperature with primary antibodies. After they were washed with TTBS for 1 hour, the membranes were incubated for 1 hour at RT with HRP-conjugated donkey anti-rabbit IgG or with goat anti-mouse IgG. Chemiluminescent signals were detected by using ECL reagent or extended-duration substrate (Supersignal West Dura; Pierce Biochemicals), and images were acquired (Super RX Film; Fuji Photofilm, Tokyo, Japan).

Immunoprecipitation

Equal amounts of cell lysate supernatants were incubated with antibody and protein A-Sepharose beads for 4 hours at 4°C for immunoprecipitation. The beads were washed three times with ice-cold lysis buffer. Bound materials were eluted with SDS-PAGE sample buffer, resolved on SDS-PAGE, transferred onto nitrocellulose membranes, and analyzed via immunoblot analysis for proteins of interest. The samples were precipitated with antibodies against FAK, paxillin, and Src. The antibodies against FAK, phospho-FAK, paxillin, phospho-paxillin, Src, and phospho-Src were used for subsequent immunoblot analysis. The phosphorylation states of these proteins were examined with antibodies against the phospho-specific forms of these components.

Rho-GTPase Activity Analysis

RhoA activity was analyzed by precipitation of the active form of RhoA (RhoAGTP) with Rhotekin Rho binding domain (RBD) beads and immunoblotting with anti-Rho antibody. In this assay, the GST-tagged RBD fusion protein associates with glutathione beads; these GST-RBD beads specifically bind to GTP-Rho but not GDP-Rho. For this assay, confluent corneal epithelial cells cultures in 100-mm culture dishes were wounded with multiple scratches with a plastic pipette tip and then treated with smoke solution for 18 hours. The cells were washed with ice-cold TBS and lysed in RIPA buffer. The cell lysates were cleared by centrifugation at 13,000 rpm at 4°C for 10 minutes and incubated with GST-RBD beads at 4°C for 1 hour. The beads were washed four times with ice-cold lysis buffer. The samples were subjected to SDS-PAGE. Both the total and bound active RhoA were...
detected by immunoblot analysis using anti-RhoA antibody. The amount of RBD-bound active RhoA was compared with the total amount of Rho in cell lysates.

F-Actin Staining in the Presence of Constitutively Active RhoA

A plasmid containing constitutively active RhoA (RhoA-V14) was transfected into corneal epithelial cells using the calcium phosphate
In the chamber was exhausted with a vortex fan. A smoking dose of it was puffed through a tube into the mouse exposure chamber; the air seconds/puff, with each puff containing 35 mL of smoke, and only 1 sitivity of Kentucky reference cigarette code1R3F, rate of smoking is 2 box was used to generate smoke according to FTC guidelines (Univer-

Absence of SSW Smoke Treatment
during Wound Healing in the Presence and

SSW smoke–exposed mice were given 1 drop every 6 hours of PBS, Tβ4 (5 μg/5 μL in PBS), and Tβ4 (5 μg/5 μL in PBS) plus dexamethasone (Dex; 1 mg/mL in PBS) solutions instilled in the eye. A customized smoking system (Teague Enterprise, Davis, CA) was used to expose the mice to SSW smoke intermittently.29,31,32 Briefly, a puffer box was used to generate smoke according to FTC guidelines (University of Kentucky reference cigarette code1R3F. rate of smoking is 2 seconds/puff, with each puff containing 35 mL of smoke, and only 1 puff per minute). The smoke produced was mixed with fresh air before it was puffed through a tube into the mouse exposure chamber; the air in the chamber was exhausted with a vortex fan. A smoking dose of 25 ± 2 mg/m³ total particulate matter (TPM) was used, to keep the exposure brief and avoid generalized physiological changes. At various times after wounding (0, 24, and 48 hours), the wounded areas were stained with sodium fluorescein and photographed. The area of each epithelial defect was quantified and analyzed (ImageJ software; Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The results were analyzed by ANOVA with the Tukey-Kramer multiple comparison test.

Chemotaxis Assays
Polycarbonate membranes with 8-μm pore size (Transwell; BD Bio-

RESULTS
Effect on HCEC Migration and Actin Polymerization
To determine whether second-hand cigarette smoke affects corneal epithelial cell migration, we used a human corneal epithelial cell line, HCECs, in both the cloning ring migration assay and the scratch-wound assay. This cell line is immortalized with SV40 and has been extensively characterized.25 The cells are cobblestone-shaped and similar to primary corneal epithelial cells in culture. By transmission electron microscopy, the authors showed that these are epithelial cells; they form desmosome and develop microvilli. They express cornea-specific 64-kDa cytokeratin in addition to five major insoluble proteins, including 8.71 IU/mg of aldehyde dehydrogenase activity. Furthermore, when this cell line was cultured at the air-liquid interface on collagen type I gels, it differentiated in a multilayered fashion.

HCECs were plated within cloning rings and allowed to adhere for 3 hours. The rings were then removed, the perimeter of the circle marked, and the cells treated with SSW smoke

mounted into the wells of a 24-well plate, such that the seeded cells were facing the bottom chamber. The medium was added to both chambers: 100 μL in the top chamber and 500 μL in the bottom chamber. To the top chamber medium we added Tβ4 (1 μg/mL), Tβ4 (1 μg/mL) + SSW (1:10), or medium alone (control). The cells were allowed to migrate toward the chemoattractant for 4 hours at 37°C. The cells on the underside of the membrane were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. The cells were counted in eight fields per filter at 20× magnification, to obtain the average number of cells per field.
solution for 18 and 40 hours. At this concentration, smoke components do not cause cell death, as confirmed with annexin V cell apoptosis assays (data not shown). At successive time intervals, we determined cell migration by measuring the distance from the initial cell edge to the migrating front. SSW smoke solution substantially decreased both cell migration and speed of migration (Figs. 1A–F). For the scratch assay, the cells were allowed to grow to confluence and then were scratched, to create a “road” devoid of cells and then exposed to SSW smoke solution for 18 hours (Figs. 1G, 1H). This assay confirmed the findings with the cloning ring assay. That is, SSW decreased corneal epithelial cell migration. This decrease in cell migration was not due to a decrease in proliferation induced by SSW treatment. Both immunolabeling for Ki67, a marker of cell proliferation, and the CCK8 assay kit which also measures cell proliferation, showed that SSW did not affect the proliferation of these cells (Fig. 2).

Cytoskeletal reorganization plays an important role in cell migration by promoting the formation of cellular processes and stress fibers that are critical for migration. To investigate the effects of SSW smoke on cytoskeletal reorganization, we examined the cells at the wound’s edge in the scratch-wound assay. After 18 hours in culture, the epithelial cells both away from the edge and, along the wound’s edge in untreated cultures, showed pronounced rhodamine-phalloidin staining with actin filaments well visible (Figs. 3A–C). In contrast, epithelial cells in cultures exposed to SSW smoke solution showed virtually no actin filament staining, both away from the edge and along the edge of the wound, indicating that SSW smoke affected actin filament polymerization (Figs. 3D–F). To exclude the possibility that lack of actin filament formation in the SSW smoke-treated cells results from the presence of a smaller pool of actin monomers in the cell, we examined the total actin in control and SSW smoke–exposed cultures by using immunoblot analysis. There were no differences in the quantity of β-actin in control and SSW smoke–treated cells, suggesting that the change in actin reorganization results from changes in signaling rather than from changes in actin protein levels (Fig. 3G).

To confirm these findings, we used an in vivo–like organ culture system, in which mouse corneas were placed in organ culture after the central epithelium was removed. The organ cultures were either left untreated or were treated with SSW smoke solution, followed by staining with rhodamine-phalloidin to visualize actin filaments. In the controls after 18 hours in culture, the usual cobblestone organization of the epithelium was interspersed with rows of cells elongated perpendicular to the wound’s edge. The cytoplasm of elongated cells showed well-developed actin cytoskeleton (Fig. 4A). Corneas of the mice exposed to SSW smoke retained their cobblestone appearance but had few, if any, elongated cells near the wound’s margin, and cells at the edge showed essentially no actin staining (Fig. 4B). To quantify these results, we measured the length and width of many cells in both the control and the smoke-treated corneas and calculated the ratio of length to width (Fig. 4C). On average, the cells in the control corneas assumed a much more elongated shape than in the SSW-treated corneas. These results suggest that SSW smoke also disrupts actin reorganization during cell migration in corneal organ cultures.

**Effect on Activation of RhoA in Corneal Epithelial Cells**

RhoA is involved in many aspects of cell migration, such as the formation of actin stress fibers and focal adhesions and membrane ruffling.15–18 Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases is mediated by specific effectors of these enzymes. To investigate the role of RhoA in corneal epithelial cell migration, we determined the levels of the activated GTP-bound form of RhoA, by using a pull-down assay with beads conjugated to a GST fusion protein of the Rho-binding domain of Rhotekin, an effector of RhoA. We found that untreated scratch-wounded cultures showed RhoA activation, whereas those treated with SSW smoke solution substantially inhibited RhoA activation (Fig. 5A). To determine the relationship between changes in RhoA activation and altered actin polymerization, we transected corneal epithelial cells with a plasmid encoding constitutively active eGFP-tagged RhoA. The transfected cells were then scratch wounded and treated with medium alone or SSW smoke solution for 18 hours, fixed, and stained with rhodamine-phalloidin. Treatment with SSW smoke inhibited actin polymerization (as shown in Fig. 3) in nontransfected cells, but not in cells transfected with constitutively active RhoA. This inhibition occurred both away from the edge (Figs. 5B, 5C) and at the edge of the wound (Fig. 5D), suggesting that inhibition of actin polymerization by SSW smoke results from smoke-mediated inhibition of RhoA activation. All cells transfected with the constitutively active RhoA and treated with SSW contained strong staining for F-actin.
Effect on the Activation of Focal Adhesion Molecules

One important signaling molecule upstream of RhoA is FAK, a member of the focal adhesion complex. Cytoskeletal changes are also associated with alterations in focal adhesion complexes.33,34 To evaluate the activation of molecules associated with focal adhesion and the formation of focal adhesion complexes, we performed reciprocal immunoprecipitation and immunoblot analyses. For these studies, confluent cultures of HCECs were scratch wounded, followed by treatment with either medium alone (control) or SSW smoke solution and Tβ4 plus SSW smoke. After 18 hours in culture, the migration was measured and photographed from the initial edge of the wound to the new cell edges. Tβ4 significantly increased corneal epithelial cell migration (C) and, in the presence of SSW smoke solution, it reversed the smoke’s inhibitory effects (D compared with A). (E) Quantitative image analysis of the distance the cells migrated in 18 hours. Statistics are shown as comparisons between the treatments with SSW smoke and the other three groups (**P < 0.05). (F) In a chemotaxis assay of HCECs, the cells were plated on the underside of the polycarbonate membranes (8-μm pore). The top chamber of the filter contained serum-free medium only, or Tβ4, or Tβ4 + SSW. After a 4-hour incubation, the cells on the underside of the membrane were removed, and the cells that migrated to the top side of the membrane were stained with toluidine blue. Cell counts were recorded in many high-power fields and then averaged. Tβ4 was chemotactic to corneal epithelial cells (**P < 0.01).

Effect on the Activation of Focal Adhesion Molecules

One important signaling molecule upstream of RhoA is FAK, a member of the focal adhesion complex. Cytoskeletal changes are also associated with alterations in focal adhesion complexes.33,34 To evaluate the activation of molecules associated with focal adhesion and the formation of focal adhesion complexes, we performed reciprocal immunoprecipitation and immunoblot analyses. For these studies, confluent cultures of HCECs were scratch wounded, followed by treatment with either medium alone (control) or SSW smoke solution. The cells were lysed, and the lysates were subjected to immunoprecipitation with the anti-FAK antibody and analyzed by immunoblot with anti-FAK and anti-phospho-FAK against Tyr 397. The phosphorylation of FAK on tyrosine 31 was also inhibited in the smoke-treated cells (Fig. 6B), similar to the observed decreases in FAK phosphorylation on tyrosine 397 (compare with Fig. 6A). In addition, FAK dissociated from a paxillin complex after SSW smoke treatment (Fig. 6B). These results suggest that SSW smoke inhibits the activation of FAK and paxillin. Because these signaling molecules are important in focal adhesion complex formation and the reorganization of actin cytoskeleton, these results may explain, at least in part, the changes in adhesion and actin reorganization after smoke treatment.

We also found that treatment with SSW smoke solution changed the distribution of the activated form of Src, a non-receptor tyrosine kinase (Fig. 7). For this assay, confluent corneal epithelial cell cultures in 100-mm culture dishes were wounded with multiple scratches with a plastic pipette tip and then treated with smoke solution for 18 hours. Although the total activated phosphorylated form of Src was not affected by SSW smoke
treatment (Fig. 7A), the localized presence of activated Src at the leading edge of the cells was inhibited by treatment with SSW smoke solution (Figs. 7B–D). Src is known to be activated after FAK and paxillin activation and to become part of the complex. In SSW-treated cells, Src was primarily found in the cytosol; very little activated src was found at the leading edge. These data suggest that, in the presence of SSW smoke, molecules in the focal adhesion complex do not interact normally.

Another molecule that is directly involved in focal adhesion formation is vinculin. We tested the possibility that vinculin expression was affected by the smoke treatment. Indeed, we found that, at the edge of the wound, much like Src, the levels of vinculin were the same in the control and in the SSW-treated cells and that the difference resides in the localization of the molecule. Like Src, vinculin localized to the leading edge in the control, but in the smoke-treated cells, it was dispersed throughout the cytosol (Figs. 7E–H).

**Effect of Tβ4 on HCEC Migration in the Presence of SSW Smoke**

Recently, it has been shown that Tβ4 can promote corneal epithelial wound healing through the stimulation of cell migration. To determine whether Tβ4 can accelerate epithelial cell migration in the presence of SSW smoke, we treated cornea scratch-wounded cell cultures with Tβ4 in the presence and absence of SSW smoke solution. Tβ4 has been shown to stimulate keratinocyte chemotaxis, although, for this assay, a much lower concentration must be used. Chemotaxis assays, by their nature, require a lower concentration of the chemotactic agent to avoid receptor desensitization. At several time intervals, we determined changes in cell migration by measuring the distance from the initial cell edge to the migrating edge. Tβ4 significantly accelerated cell migration compared with the control. It also significantly increased the ability of the cells to migrate, even in the presence of SSW smoke solution (Figs. 8A–E). To determine whether the Tβ4-induced migration is a result of directed movement, we performed chemotaxis assays and found that Tβ4 stimulated corneal epithelial cell directed migration when compared with that in the control cells. Furthermore, when in the presence of SSW, Tβ4 reversed the smoke’s effects on migration, so that the cells were chemotactically similar to the control cells (Fig. 8F). These data correlate well with the scratch assay shown in Figures 8A–E.

We also used time-lapse video microscopy to illustrate the differences seen with Tβ4 treatment (Fig. 9). The control cells move by extending lamellipodia at the edge of the wound with adhesion plaques evident at the edge of the ruffles (Fig. 9A). When the cells were treated with Tβ4, these effects were much more pronounced with virtually the whole cell, except for the trailing edge, involved in migration (Fig. 9C). Treatment with SSW caused the cells to lose the ability to extend lamellipodia (Fig. 9B), but when the cells were also treated with Tβ4, they were able to extend them as much as the control (Fig. 9D; compare with 9A). These results correlate well with the results presented in Figure 8. Both in the chemokinesis and the chemotactic assays, Tβ4 reversed the SSW inhibition of corneal epithelial cell movement. We further stained cells at the edge of the wound with vinculin to illustrate the changes in vinculin localization. The control cells at the edge of the wound showed a significant amount of vinculin localized at the lamellipodia edge (Fig. 7F), whereas in the SSW-treated cells, vinculin was primarily localized in the cytosol (Fig. 7G). When the cells were treated with Tβ4, most of the vinculin was localized at the edge of the lamellipodia in the focal adhesion plaques (Fig. 9E). When the SSW cells were treated with Tβ4, we saw a recovery of the localization of vinculin to the lamellipodia (Fig. 9F) equal to the level in the control (Fig. 7F).

**Effect of Tβ4 on Corneal Epithelial Wound Healing**

To determine whether Tβ4 can reverse the inhibition on corneal healing induced by SSW smoke, we treated mice exposed to smoke with Tβ4. After the corneas were wounded, the mice were exposed to cigarette smoke emitted by a smoking apparatus that mimics second-hand cigarette smoke. Corneal epithelial wound healing in Tβ4-treated mice was similar to that of the control mice, which were treated with vehicle alone (Figs. 10A, 10B, 10E, 10F). In addition, Tβ4 increased wound closure in mice exposed to SSW smoke but did not completely reverse the smoke’s effects (Figs. 10A–D, 10G–H). We know that inflammation is also a critical component of SSW smoke–induced delay in corneal healing and that Dex, a strong anti-inflammatory agent, partially re-
verses the SSW-induced inhibition of healing. Therefore, we hypothesized that treatment with both Tβ4 and Dex would have an additive effect on healing. Indeed, mice with wounded corneas exposed to SSW smoke and treated with both Tβ4 and Dex exhibited healing comparable to that in the untreated controls (Figs. 10I, 10J, 10K). However, it has been shown that human corneal epithelial cells have a receptor for Dex and treatment with Dex increases cell proliferation. To determine whether the effects of Dex could be related to the increase in proliferation of the corneal epithelial cells, we performed a proliferation assay with the CCK8 kit. As shown in Figure 11, SSW Dex did not stimulate cell proliferation, and neither did any of the other treatments used to stimulate the rate of healing in the cornea. We conclude that SSW smoke impairs corneal wound healing through both increased inflammation and decreased epithelial cell migration.

**DISCUSSION**

In this study, we delineated potential mechanisms involved in the effects of second-hand cigarette smoke on corneal wound healing. SSW smoke (1) inhibited corneal epithelial cell migration and reorganization of the actin cytoskeleton in both cell and organ cultures; (2) inhibited RhoA, an important component that regulates actin cytoskeleton reorganization; introduction of constitutively active RhoA reversed the effects of smoke on the actin cytoskeleton; and (3) inhibited the phosphorylation/activation and redistribution of FAK, paxillin, Src, and vinculin, all components of focal adhesion complexes. Furthermore, Tβ4 stimulated both corneal epithelial cell migration in vitro and closure of the corneal epithelial wounds in vivo in the presence of SSW smoke exposure; and that, in combination with Dex, reversed the SSW smoke-induced inhibition of healing. These findings show that SSW smoke exerted its effects on cell migration during corneal epithelial wound healing through the inhibition of actin reorganization and the inactivation of focal adhesion molecules and Rho-GTPases.

The cell migration studies in both cell and organ cultures provided strong evidence of the adverse effects of SSW smoke on corneal epithelial cell migration. Staining of F-actin with rhodamine-phalloidin in both organ and cell cultures showed similar results—that is, weak F-actin staining of the cellular processes and cell bodies in the cells exposed to SSW smoke.

**FIGURE 10.** Effects of Tβ4 and Dex on corneal epithelial wound healing in mice exposed to SSW smoke. (A-J) Representative images of the corneal epithelial wound healing process in the presence of various treatments. (A, B) Wounded corneas from mice not exposed to SSW smoke (exposed to PBS); (C, D) corneas of mice exposed to SSW smoke (diluted in PBS) only; (E, F) corneas of mice treated with Tβ4 (diluted in PBS) only; (G, H) corneas of SSW smoke-treated mice with application of Tβ4 (diluted in PBS); (I, J) corneas of SSW smoke-exposed mice treated with both Tβ4 and Dex (diluted in PBS). Red circles: the initial wound edges; gray circles: edges of the wounds at the indicated time points. (K) Statistical analysis shows that Tβ4 improved wound-healing in mice exposed to SSW smoke, whereas Tβ4 + Dex reversed SSW smoke-induced inhibition of healing. The areas of wounds were quantified and analyzed with Image J. ANOVA with Tukey-Kramer multiple-comparisons test was performed. Statistics are shown as comparisons between the treatment of SSW smoke and the other three groups. ***P < 0.05, n = 6 in each group.

**FIGURE 11.** Effects of SSW, Tβ4, Dex, and combinations of treatments on the proliferation of HCECs. The CCK8 assay was used to determine the effect on cell proliferation of the various treatments alone or in combination. No significant changes were detected.
compared with that in the untreated control cells. As the immunoblot analysis results show, the overall protein levels of β-actin were not changed by SSW smoke exposure, suggesting that smoke alters the regulation of actin dynamics, reorganization, and/or F-actin formation.

Because of the known role of RhoA in the regulation of actin cytoskeletal reorganization, we investigated the effects of SSW smoke on RhoA activation. Using the RhoA-GTP pull-down assay, we found that SSW smoke inhibits the activation of RhoA. When corneal epithelial cells were transfected with plasmids encoding constitutively active RhoA, polymerized actin fibers reappeared in the migrating cells along the scratch wound, even in the presence of SSW smoke. The results of these experiments suggest that the observed defects in actin polymerization after smoke exposure result from smoke inhibition of RhoA activation.

Because of the relationship between FAK and RhoA in the reorganization of actin filaments, we also determined changes in activation of focal adhesion complexes during cell migration. Co-precipitation of FAK and paxillin showed that the phosphorylation of these two molecules and their interactions were inhibited by SSW smoke exposure. Because of the close relationship between FAK and Src, we also determined the activation and distribution of Src. Immunoblot analysis showed that phosphorylated Src levels are not altered by SSW. However, immunolabeling showed that the localization of phosphorylated Src in focal adhesion complexes was inhibited by SSW smoke in cells along the wound’s edge. We observed the same effects with vinculin. The data described herein show the impact on and potential mechanisms of SSW smoke exposure in corneal epithelial cell migration. Indeed, RhoA has been associated with events of cell migration that could lead to the development of leader cells at the edge of the wound; this could be influential in the migratory behavior of the adjacent cells.36 However, in our time-lapse video microscopy, we never observed the formation of leader cells at the migrating edge.

Our previous study7 showed that SSW smoke inhibits corneal epithelial wound healing in vivo through the stimulation of inflammation. When we inhibited smoke-induced inflammation using either anti-inflammatory drugs or neutrophil depletion, healing was significantly improved. However, the wounds still did not heal normally. This new study demonstrates that cigarette smoke may also impair corneal epithelial wound healing through the inhibition of cell migration. To determine whether stimulation of cell migration can reverse the inhibitory effect of SSW smoke on corneal wound healing in vivo, we performed cell migration and in vivo assays, in the presence of SSW smoke and Tβ4. Tβ4 is primarily known for its ability to bind actin monomers. However, more recently it has been shown to stimulate cell migration,21–23 but the mechanisms by which it stimulates migration are currently unknown. We also found that Tβ4 partially reversed the inhibitory effect of SSW smoke on corneal epithelial cell migration in vitro and corneal wound healing in vivo. Furthermore, corneas exposed to SSW smoke and treated with both Tβ4 and Dex exhibited healing comparable to that in untreated control corneas, suggesting that SSW smoke delays corneal wound healing through both increased inflammation and decreased cell migration. These results support previous findings that showed that Tβ4 stimulates keratinocyte chemotaxis.34 Furthermore, anti-inflammatory agents stimulate production by monocytes of Tβ4 which also acts as an inhibitory signal for the inflammatory response.25 We are currently using our system and a variety of cellular and molecular approaches to unravel the mechanisms by which Tβ4 stimulates cell migration and how these two effects are linked and hence can explain the reversal of the cigarette smoke delay of healing of corneal epithelium.

In summary, the studies described herein show the impact and potential mechanisms of exposure to second-hand cigarette smoke on corneal epithelial wound healing. Individuals with corneal epithelial injuries exposed to second-hand cigarette smoke may have impaired healing due to defective cell migration and inflammation in the early stages of healing. Our results also suggest a potential for the combined use of anti-inflammatory and promigratory agents to promote wound healing.

Acknowledgments

The authors thank Suzanne J. Fleiszig (University of California, Berkeley) for providing human corneal epithelial cells, colleagues in our laboratory for helpful discussions, and Melissa Petreca for reading the final version of the manuscript.

References


