Substance P Enhances Keratocyte Migration and Neutrophil Recruitment through Interleukin-8

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ABSTRACT

Keratocytes, the resident cells of the corneal stroma, are responsible for maintaining turnover of this tissue by synthesizing extracellular matrix components. When the cornea is injured, the keratocytes migrate to the wounded site and participate in the stromal wound healing. The neuropeptide substance P (SP), which is also known to be produced by non-neuronal cells, has previously been implicated in epithelial wound healing after corneal injury. Corneal scarring, which occurs in the stroma when the process of wound healing has malfunctioned, is one of the major causes of preventable blindness. This study aimed to elucidate the potential role of SP in keratocyte migration and therefore in stromal wound healing. We report that the expression and secretion of SP in human keratocytes are increased in response to injury in vitro. Moreover, SP enhances the migration of keratocytes by inducing the actin cytoskeleton reorganization and focal adhesion formation through the activation of the phosphatidylinositide 3-kinase and Ras-related C3 botulinum toxin substrate 1/Ras homolog gene family, member A pathway. Furthermore, SP stimulation leads to upregulated expression of the proinflammatory and chemotactic cytokine interleukin-8 (IL-8), which also contributes significantly to SP-enhanced keratocyte migration and is able to attract neutrophils. In addition, the preferred SP receptor, the neurokinin-1 receptor, is necessary to induce keratocyte migration and IL-8 secretion. In conclusion, we describe new mechanisms by which SP enhances migration of keratocytes and recruits neutrophils, two necessary steps in the corneal wound-healing process, which are also likely to occur in other tissue injuries.

Introduction

The cornea is the transparent front part of the eye and is responsible for most of the eye's total optical power. It comprises three functionally distinct layers: an outer epithelial layer, a middle stromal layer, and an inner endothelial layer. The stroma accounts for up to 90% of the corneal thickness, and it consists of regularly packed collagen fibrils arranged into lamellae (Maurice, 1957; Benedek, 1971; Farrell et al., 1973; Hassell and Birk, 2010; Ruberti et al., 2011). Sparsely distributed between the lamellae, the quiescent cells of the stroma, keratocytes, are found. They have a dendritic morphology and are responsible for maintenance and repair of the stroma. Keratocytes produce lumican and keratocan, which regulate corneal transparency and shape (Muscelmann et al., 2005). The cornea is protected from injury both by physical and molecular barriers; however, under some circumstances, such as trauma, infection, or surgery, the cornea may get injured. When the cornea gets injured a healing response is activated, which ideally leads to a healed, healthy cornea (Lim et al., 2003). The corneal wound-healing process is a complex interplay between the epithelium, the stroma, and the immune system, and it is mediated by various cytokines and growth factors. Briefly, after an injury occurs, keratocytes adjacent to the wound undergo apoptosis. As apoptosis results in an area devoid of keratocytes, the remaining cells start to proliferate and migrate toward the wound. Some of the mitotic keratocytes generate myofibroblasts, which are mainly responsible for producing new extracellular matrix components (Wilson et al., 2001). At 12–24 hours after injury, there is an
influx of inflammatory cells to the stroma (O’Brien et al., 1998). Cells such as macrophages, monocytes, and neutrophils scavenge cellular components released from dying keratoocytes. In the final stage of corneal wound healing, restoration of the quiescent state of the keratoocytes and remodeling of any disordered collagen take place (Wilson et al., 2001). When the process of the wound healing is incomplete or disturbed, corneal scarring may occur, transforming the transparent cornea to an opaque tissue with consequent visual impairment. Despite extensive research on corneal wound healing, corneal scarring remains a major cause of preventable blindness (Stepp et al., 2014). More in-depth understanding of this process is still needed.

The migration of keratoocytes is an important step toward proper wound closure and healing. The process of migration involves reassembly of the actin cytoskeleton, and in this step, a small G protein called Ras-related C3 botulinum toxin substrate 1 (Rac1) is one of the main regulators (Ridley, 2001). Moreover, phosphoinositide 3-kinases (PI3Ks) are the key players in maintaining cell polarity and defining the leading edge of the migrating cell (Cain and Ridley, 2009). Well-coordinated cooperation of Rac1, Ras homolog gene family, member A (RhoA), and PI3Ks is needed for a successful cell migration and thus wound closure.

Substance P (SP) is a neuropeptide well-known for its role in the sensory nervous system, including that it has been detected in sensory nerve fibers of the cornea (Muller et al., 2003). Nevertheless, it has been shown that SP can also be produced by non-neuronal cells, such as corneal epithelial cells and keratoocytes (Watanabe et al., 2002). SP acts through binding to G protein–coupled receptors; the neurokinin 1 receptor (NK-1R) is the subtype with the highest affinity for SP (Regoli et al., 1994). SP activates the phospholipase C (Nakajima et al., 1992), protein kinase C, and PI3K (Sun et al., 2009) signaling pathways to initiate diverse physiologic functions such as modulation of immune cell activity (Maggi, 1997), vasodilation of blood vessels (Baluk et al., 1997), and contraction of smooth muscles (Lundberg and Saria, 1982). Moreover, SP has been shown to sensitize corneal epithelial cells to migrate (Yamada et al., 2005; Yang et al., 2014) and to stimulate synthesis of the proinflammatory cytokine interleukin 8 (IL-8), which recruits and activates neutrophils during inflammation (Hammond et al., 1995; Tran et al., 2000). Importantly, studies have demonstrated that increased SP levels accelerate corneal wound healing in an alkali burn model in rabbits (Hong et al., 2009). The samples were then transferred and cultured in DMEM/F12 medium supplemented with 2% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco). Medium was replaced every second to third day until the cells reached confluence. Confluent cells were detached with 0.05% trypsin-EDTA (Gibco) and split in a 1:2 ratio. Cells from the central cornea in passages 4 to 5 were used for experiments. Corneas were assessed individually.

Cell Culture and Treatments. To maintain keratocyte phenotype, central keratoocytes were cultured in DMEM/F12 medium supplemented with 2% FBS (instead of 10% FBS, which transforms keratoocytes into fibroblasts and activates them) and 1% penicillin-streptomycin. Culturing keratoocytes in medium supplemented with 2% FBS maintains their proper phenotype: cells express keratocan and lumican, markers of keratoocytes, and only small amounts of α smooth muscle actin muscle actin (α-SMA), a marker of activated keratoocytes (Stoliecka et al., 2015). For the experiments, cells were seeded in either six-well plates (Sarstedt, Helsingborg, Sweden): 0.25 × 10^6 cells/well [Western blot and reverse transcription (RT)-quantitative polymerase chain reaction (qPCR)], 0.4 × 10^6 cells per well (scratch and wound-healing assay), 1 × 10^6 cells/well (scratch assay for SP enzyme immunoassay, ELISA) or in 96-well plates (Sarstedt): 8000 cells/well (IL-8–enzyme-linked immunosorbent assay, ELISA) in DMEM/F12 medium containing 0.1% FBS (unless stated otherwise) and allowed to adhere overnight. Vehicle controls (DMEM for wortmannin [concentration 0.00495%], water for SP and L-733,060; referred to as controls, as no differences between controls and vehicle controls were found) were included. To block NK-1 receptors, cells were incubated with L-733,060 (Tocris, Bristol, UK) for 30 minutes at 37°C. Afterward, cells were treated with two different concentrations of SP: 10^{-8} M and 10^{-7} M (Sigma). To confirm IL-8 induced migration, either IL-8–neutralizing antibody (3 μg/ml; Pepro Tech, Rocky Hill, NJ) was added to the cells 8 hours after SP treatment, or cells were treated with various concentrations of recombinant human IL-8 (Pepro Tech). Normal rabbit IgG antibody was used as an isotype control (R&D Systems, Minneapolis, MN) at a concentration of 3 μg/ml. Wortmannin (Sigma) was used to inhibit PI3K. Cells were pretreated with 100nM wortmannin for 1 hour at 37°C; afterward, they were treated with SP. Various concentrations of L-733,060, SP, and wortmannin were tested regarding the effect on keratocyte viability, and concentrations that were nontoxic to the cells were chosen for the subsequent experiments. Interleukin 8 receptor α (CXCR1) and interleukin 8 receptor β (CXCR2) antibodies (MR&D Systems) were used to block IL-8 receptors. Cells were incubated with 2 μg/ml of CXCR1 and CXCR2 antibodies for 1 hour at 37°C. Mouse IgG2A antibody was used as an isotype control (R&D Systems) at a concentration of 2 μg/ml.

Materials and Methods

Collection of Human Corneas. Healthy human corneas from deceased patients who had chosen, when alive, to donate their corneas postmortem for transplantation and research, according to Swedish law, were kept in a corneal biobank at the University Hospital of Umeå, Sweden. If these healthy donated corneas were not used for transplantation after their collection, they were delivered to the laboratory for research purposes. If corneas were used for transplantation, some or all of the transplantation graft leftovers were retrieved for study purposes: the donor healthy limbal part or the donor healthy anterior or posterior central lamella. The study was vetted by the Regional Ethical Review Board in Umeå (2010-373-31M) without objections. The study was performed according to the principles of the Declaration of Helsinki.

Isolation and Primary Culture of Human Keratoocytes. Healthy human corneas were obtained from donated transplantation grafts or graft leftovers, as described in the previous section. Primary culture of human keratoocytes was established. Samples were scraped using a sterile scalpel to remove any remaining epithelial or endothelial cells before being washed in sterile Hank’s balanced salt solution (HBSS no. 14170; Invitrogen, Carlsbad, CA). In cases of whole corneal samples, the central part was separated from the peripheral part using a scalpel. Each part was then minced with a scalpel and digested in 2 mg/ml collagenase (clostridiopeptidase A, no. C-1030; Sigma, St. Louis, MO) diluted in Dulbecco’s modified Eagle’s medium (DMEM) nutrient mixture F-12 (F-12-095; Gibco, Carlsbad, CA) at 37°C. The samples were then transferred and cultured in DMEM/F12 medium supplemented with 2% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (Gibco). Medium was replaced every second to third day until the cells reached confluence. Confluent cells were detached with 0.05% trypsin-EDTA (Gibco) and split in a 1:2 ratio.

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Keratocytes were fixed with 3.7% paraformaldehyde in 1:8 well chamber slides (Corning, Corning, NY). After treatments, cells were centrifuged at 250 RCF for 5 minutes and washed with blood cells were lysed with red blood cell lysis buffer. Afterward, cells were centrifuged at 250 RCF for 5 minutes and washed with HBSS without Ca²⁺/Mg²⁺ twice. Neutrophils were counted, adjusted to the desired concentration, and used within 2 hours after isolation. Isolated neutrophils were primed with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich).

**RT-qPCR Assay.** IL-8, TAC1 (SP), and TACR1 (NK-1R) mRNA levels were assessed by RT-qPCR. Total RNA was isolated from keratocytes by RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol and reverse transcribed into cDNA with High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). To determine IL-8, TAC1 and TACR1 gene expression, TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) were used. cDNA transcribed from 40 ng of RNA was run in duplicates by Viia 7 Real-Time PCR system (Applied Biosystems), with 18S as an internal control (Life Technologies), and analyzed with Viia 7 Software (Applied Biosystems).

**Western Blot Analysis.** Keratocytes were washed with phosphate-buffered saline (PBS) and freeze/thawed three times. Cells were further lysed in radioimmunoprecipitation assay buffer supplemented with 0.5% proteinase inhibitor cocktail (Sigma). Protein concentration was assessed by Bradford assay (BioRad, Hercules, CA). Total proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked with 5% (w/v) BSA (Sigma) or 5% nonfat milk in TRIS-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies: anti-NK-1R (R&D Systems, Minneapolis, MN), anti-RhoA, anti-SAPK/JNK, anti-phospho-SAPK/JNK (Thr183/ Tyr185), anti-NF-κB p65, anti-phospho-NF-κB p65 (Ser536), and anti-β-actin (Cell Signaling, Leiden, The Netherlands), and anti-CXCR1 and anti-CXCR2 (Abcam, Cambridge, UK). After washing, horseradish peroxidase–conjugated secondary antibody was added and incubated for 1 hour at room temperature. Detection of Rac1, membranes were incubated with Rac1 antibody (Cytoskeleton, Denver, CO) for 1 hour at room temperature in TBS-T, washed, and incubated with horseradish peroxidase–conjugated secondary antibody for 30 minutes at room temperature in TBS-T. Images were taken by Odyssey Fc imaging system (LI-COR, Lincoln, NE). Densitometry was performed using Image J analysis software (National Institutes of Health). Densitometry analysis of phosphorylated NF-κB and SAPK/JNK was calculated as follows: intensity of the phosphorylated protein of interest was divided by the intensity of β-actin. Intensity of total protein of interest was divided by the intensity of β-actin. Ratio of phosphorylated protein of interest was divided by ratio of the total protein of interest.

**ELISA/EIA.** Supernatants collected from scratch assay were subjected to SP EIA kit (Phoenix Pharmaceuticals, Burlingame, CA) to measure SP secretion, according to the manufacturer's protocol. IL-8 secretion was measured with human CXCL8/IL-8 DuoSet (R&D Systems) according to the manufacturer's protocol.

**Immunofluorescence Staining.** We seeded 10⁴ cells/well in eight-well chamber slides (Corning, Corning, NY). After treatments, medium was removed and cells were washed twice with PBS. Keratocytes were fixed with 3.7% paraformaldehyde in 1× PBS for 20 minutes at room temperature, permeabilized with 0.1% Triton X-100 in 1×PBS for 5 minutes, then blocked for 30 minutes with 1% BSA in PBS containing 0.1% Tween-20 (PBS-T). Cells were incubated with anti-vinculin antibody (Sigma) for 1 hour. After washing, secondary antibody labeled with tetramethylrhodamine (Dako, Glostrup, Denmark) was added together with BODIPYFL phallacidin (Life Technologies) for 30 minutes. Finally, cells were mounted in ProLong Diamond Antifade Mountant with DAPI (Life Technologies). A control well was also prepared for secondary antibody by replacing the primary antibody with PBS. A Zeiss Axioskop 2 plus microscope equipped with epifluorescence and an Olympus DP70 digital camera were used for analysis.

**Scratch Assay.** The 10⁴ keratocytes were seeded into six-well plates in DMEM/F12 medium supplemented with 0.1% FBS and allowed to adhere overnight. Next morning, medium was replaced with fresh one, and the bottom of the well was scratched with a 200-μl pipette tip, leaving the control wells not scratched. Cells were washed with PBS, and fresh medium was added. Supernatants were collected (for SP EIA) at 8 and 24 hours, and cells were lysed in lysis buffer (for TAC1, TACR-1 RT-qPCR) at 8 hours.

For the migration scratch assay, 0.4 × 10⁶ keratocytes were seeded into six-well plates in DMEM/F12 medium without FBS and allowed to adhere overnight. The next morning, medium was replaced with fresh one, and the bottom of all wells was scratched with a 200-μl pipette tip, creating a wound field. Cell were washed with PBS and treated as described in Cell Culture and Treatments section. Images of the wounds were taken at same spots, at times 0, 8, 24, and 48 hours using Motic AE31 Trinocular inverted microscope (Richmond, BC, Canada). Cell migration was quantified by counting cells that migrated into the created wound field at 0, 8, 24, and 48 hours and compared with 0 hours (the number of cells that had migrated into the wound field at a specified time point was divided by the number of cells that were in the wound field at time 0). Counting was done with ImageJ software.

**Chemoatxis.** CytoSelect 24-Well Cell Migration Assay (Cell Biologs, San Diego, CA) (transwell assay) was used to assess SP-induced chemotaxis, involvement of PI3K in SP-enhanced migration, and the role of IL-8 in keratocyte migration. Isolated human neutrophils and keratocytes were used as migratory cell populations. Two different membrane pore sizes were used: 3 μm for neutrophils and 8 μm for keratocytes. The assay was used according to the manufacturer's protocol. Briefly: 500 μl of supernatants collected from cell treatments or fresh medium supplemented with SP or different concentrations of IL-8 was added to the lower well of the migration plate. Suspension of either 1.5 × 10⁵ neutrophils or 0.4 × 10⁶ keratocytes in serum-free medium was added to the inside of each insert. Plates were incubated for 18 hours in a cell culture incubator. After incubation time, media from the inside of the insert were pirated and the insert transferred to a clean well containing Cell Detachment Solution (Cell Biologs) and incubated for 30 minutes at 37°C. Cells were dislodged from the underside of the membrane by tilting the insert several times in the detachment solution. Medium from the lower well of the plate was transferred to the well containing Cell Detachment Solution for the same migration assay sample (only neutrophils assay); 180 μl of the mixture was transferred to a 96-well plate and treated with lysis buffer and CyQuan GR dye solution for 20 minutes at room temperature. Mixture was transferred into 96-well plate suitable for fluorescence measurement (Corning) and fluorescence was read with a Synergy HT plate reader at 485/520nm (BioTek, Winooski, VT).

**Cell Proliferation Assay.** Three thousand keratocytes per well were seeded into 96-well plates in DMEM/F12 medium containing 0.1% FBS and allowed to adhere overnight. Cell were treated with two concentrations of SP (10⁻⁴ M and 10⁻³ M) or the following concentrations of IL-8: 0.25 ng/ml, 0.5 ng/ml, 0.75 ng/ml, 1 ng/ml, 10 ng/ml, 50 ng/ml and 100 ng/ml. Cell proliferation was measured with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Proliferation was measured at 0, 8, 24, 48, and 72 hours after stimulation.
Statistical Analysis. Statistical analyses were performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Data were expressed as means ± S.D. Statistical analysis was performed with one-way or two-way analysis of variance and Bonferroni post hoc test. A P value of <0.05 was predetermined as a statistically significant difference. All experiments were successfully performed at least three times. (i.e., at least three separate experiments were performed with cells isolated from different patients). All samples were prepared in triplicates.

Results

Scratch Injury Increases SP Production by Keratocytes Cells In Vitro. To test whether corneal injury has an effect on SP production by keratocytes, primary human keratocytes were subjected to a scratch assay. Cells were grown in six-well plates, and a scratch injury was created by using a 200-μl pipette tip. Cells were lysed 8 hours after induction of the injury and analyzed for TAC1 (SP) and TACR1 (NK-1R) gene expression quantification. Both TAC1 and TACR1 genes were upregulated after scratch injury (Fig. 1A). Moreover, supernatant from injured cells was collected and cells were lysed at 8 and 24 hours after injury. SP secretion and NK-1R protein expression were then measured by SP EIA and Western blot, respectively. The analysis revealed that the levels of secreted SP were significantly elevated 8 and 24 hours after injury (Fig. 1B). Expression of NK-1R was significantly increased at both 8 and 24 hours in cells subjected to scratch injury (Fig. 1C).

SP Increases Migration of Keratocytes In Vitro through NK-1R. To investigate the possible effect of SP on migration of keratocytes, cells were subjected to scratch injury and treated with two different concentrations of SP (10^{-7} and 10^{-5}M). To evaluate whether SP acts through its preferred receptor, NK-1R, cells were pretreated with the NK-1R antagonist L-733,060 (10^{-6}M) before the scratch injury. Migration of keratocytes was observed under microscope, and pictures were taken at 0, 8, 24, and 48 hours (Supplemental Fig. 1). Cells were counted inside the created injury and compared with time 0. Cells treated with 10^{-7}M SP showed enhanced migration toward the center of the scratch injury 24 hours after injury, and at 48 hours, both concentrations of SP significantly increased keratocyte migration (Fig. 2A). SP was shown to act through NK-1R, as the SP-induced increased migration of the cells was abolished at both 24 and 48 hours after L-733,060 pretreatment (Fig. 2A). To further quantify SP-induced migration, we used CytoSelect cell transwell migration assay. SP in fresh medium served as a chemoattractant and keratocytes as migratory cells. Cells were allowed to migrate for 18 hours. Both concentrations of SP (10^{-7} and 10^{-5}M) had a significantly positive effect on cell migration, and this effect was dose dependent (Fig. 2B). Pretreatment of keratocytes with L-733,060 (10^{-6}M) abolished the effect of SP on their migration (Fig. 2B). Additionally, SP had no proliferative effect on keratocytes (data not shown).

SP Induces Actin Cytoskeleton Reorganization and Formation of Focal Adhesion Points in Keratocytes In Vitro by Activation of PI3K. Cytoskeletal changes and formation of focal adhesion points after cells were stimulated with SP were studied. Cytoskeleton changes occurred in cells stimulated with SP (10^{-5}M) and could be observed 8 and 48 hours after stimulation. As seen in Fig. 3, in control (untreated) cells actin filaments (F-actin) were well organized and distributed throughout the cell. The control cells had no formation of focal adhesion points (vinculin), and their shape was elongated. After stimulation with 10^{-5}M SP (8 and 48 hours), spike-like filopodia were observed and lamellipodia could be found at the leading edges of the migrating cells. Prominent stress fibers appeared. The migrating cells also showed formation of numerous focal adhesion points. To confirm that SP acts through NK-1R in this regard, cells were pretreated with L-733,060 (10^{-6}M) and subsequently were treated with 10^{-5}M SP. Actin reorganization could not be observed then, the cells did not form a leading edge with lamellipodia, and no formation of focal adhesion points could be seen. The cells remained in their resting state. The same
effect was observed for SP at a concentration of 10^{-7} M (Supplemental Fig. 2).

Furthermore, we studied PI3K involvement in actin reorganization and formation of focal adhesion points (Fig. 3). The PI3K inhibitor wortmannin treatment reduced formation of actin stress fibers. The cells did not have a leading edge or filopodia or lamellipodia. A slight membrane ruffling could be observed. The cells had a spindle-like shape and did not form focal adhesion points at either 8 or 48 hours.

**SP Uregulates Rac1 and RhoA through Activation of PI3K.** The expression of Rac1 and RhoA, the two proteins involved in regulation of cytoskeleton, in keratocytes was evaluated by Western blotting at 8 and 48 hours after SP stimulation (10^{-7} M and 10^{-5} M) (Fig. 4A). The results showed that expression of both Rac1 and RhoA was upregulated in SP-treated cells at both 8 and 48 hours. When cells were pretreated with L-733,060 (10^{-8} M) before SP treatment, Rac1 and RhoA expression were at the same levels as for the control group (except for cells pretreated with L-733,060 together with 10^{-5} M SP at 8 hours, in which both Rac1 and RhoA were upregulated). To examine whether PI3K is involved in SP-enhanced migration, we treated cells with 100 nM wortmannin (a PI3K inhibitor) and SP, and we found that SP was not able to induce upregulation of either Rac1 or RhoA (Fig. 4A). To confirm PI3K importance in SP-enhanced migration, keratocytes were pretreated with 100 nM wortmannin and then subjected to chemotaxis in which SP 10^{-7} or 10^{-5} M served as chemoattractant. Cells pretreated with wortmannin and subsequently treated with SP showed significantly decreased migration compared with cells treated with SP alone (Fig. 4B). Moreover, keratocytes pretreated with wortmannin in the scratch injury model migrated significantly less when compared with cells treated with only SP at 8, 24, and 48 hours (Fig. 4C).

**SP Increases IL-8 Gene Expression and Secretion.** Because it has been reported that SP is able to stimulate cells to produce IL-8, we wanted to see whether SP would have this effect on keratocytes. First, IL-8 gene expression was assessed by RT-qPCR after stimulation with two concentrations of SP (10^{-7} and 10^{-5} M) (Fig. 5A). SP stimulation increased IL-8 gene expression in a dose-dependent manner; higher concentration was more potent. The maximum expression occurred 6 hours after stimulation, with a sharp decline at 12 hours. Next we aimed to explore the mechanism by which SP induced IL-8 expression. Two pathways that control IL-8 transcription (SAPK/JNK, NF-κB) and one pathway that controls post-transcriptional changes to IL-8 mRNA (p38 MAPK) were analyzed. SP stimulation (10^{-7} M and 10^{-5} M) led to phosphorylation of SAPK/JNK (p46) after 1 and 5 minutes. SP stimulation also led to phosphorylation of NF-κB p65, with peaks at 15 and 30 minutes for both concentrations of SP. Additionally, a second peak of phosphorylated NF-κB p65 could be observed at 180 minutes for both concentrations of SP (Fig. 5, B and C). SP stimulation did not result in p38 MAPK phosphorylation (data not shown). Next, to check whether SP stimulation leads to increased IL-8 secretion, we treated cells with two different concentrations of SP (10^{-7} and 10^{-5} M) and performed ELISA (Fig. 5D). IL-8 secretion was also dose dependent. Higher concentration of SP had a significant effect on IL-8 secretion 8 hours after stimulation, whereas lower concentration had an effect only at 48 hours. We noted a significant difference between the two concentrations used; however, 72 hours after stimulation, this difference was not observed anymore. To check whether SP-induced IL-8 secretion, cells were pretreated with two concentrations of L-733,060 (10^{-7} and 10^{-6} M) and subsequently treated with SP (10^{-7} and 10^{-5} M) for 24 hours. IL-8 secretion was measured by ELISA (Fig. 5E). The results showed that IL-8 secretion was decreased when pretreated with both concentrations of L-733,060 compared with SP alone, indicating that SP acts through NK-1R to stimulate IL-8 expression.

**IL-8-Secreted by Keratocytes after SP Stimulation Increases their Migration.** It is known that IL-8 induces cell migration. Therefore, we wanted to determine whether IL-8 secreted after SP stimulation has an effect on keratocyte migration. First, expression of IL-8 receptors CXCR1 and CXCR2 in keratocytes was confirmed by Western blot (Supplemental Fig. 3). To test whether IL-8 is involved in keratocyte migration, a scratch assay was performed. Cells were scratched with a 200-μl pipette tip and treated with 10^{-7} M and 10^{-5} M SP. Eight hours after stimulation, IL-8-neutralizing antibody or normal rabbit IgG isotype controls were added (3 μg/ml). Images were taken at 0, 8, 24, and 48 hours to observe cell migration toward the center of the scratch (Supplemental Fig. 4). Cells were counted inside the created injury and compared with time 0. Keratocytes treated with IL-8-neutralizing antibody showed decreased migration compared with cells treated with either SP or isotype control and SP. This effect could be observed at 24 and 48 hours after...
SP stimulation (Fig. 6A). CytoSelect cell transwell migration assay was used to test whether cell migration was dependent on IL-8 concentration. Keratocytes served as migratory cells. As a chemoattractant, we used supernatants collected from keratocytes not treated (control), treated with SP (10^{-7} M and 10^{-5} M), or pretreated with L-733,060 (10^{-6} M) before SP treatment. IL-8 concentrations were measured in these supernatants (Supplemental Table 1). Cells were allowed to migrate for 18 hours. Migration of keratocytes, for which supernatant collected from SP-treated cells served as a chemoattractant, was increased compared with controls. Cell migration was not, however, enhanced when supernatant from L-733,060- and SP-treated cells was used (Fig. 6B). An enhanced migration corresponded to a higher concentration of IL-8 (Supplemental Table 1). To further confirm IL-8 involvement in keratocyte migration, we used supernatants from keratocytes that were untreated or treated with SP (10^{-5} M) and/or IL-8-neutralizing antibody and/or normal rabbit IgG control (3 μg/ml) as chemoattractants. Again, IL-8 concentrations were measured in these supernatants (Supplemental Table 2). Keratocytes served as migratory cells. Migration of keratocytes was increased with supernatant collected from SP and isotype control + SP-treated cells, whereas cell migration was significantly decreased when supernatant collected from SP + IL-8 neutralization–treated cells was used as a chemoattractant (Fig. 6C). Interestingly, supernatant collected from cells treated with IL-8 neutralization antibody alone also decreased cell migration compared with supernatant of untreated cells. Again, enhanced migration corresponded to a higher concentration of IL-8. Finally, IL-8 receptors (CXCR1 and CXCR2) were blocked on keratocytes, and these cells were allowed to migrate toward supernatant collected from cells treated with SP (10^{-5} M) (in which IL-8 concentration was measured) (Supplemental Table 3). Blockage of IL-8 receptors significantly decreased cell migration compared with cells with IL-8 receptors not blocked or cells treated with mouse IgG2A isotype control, indicating that IL-8 is partially involved in SP-induced migration (Fig. 6D). Moreover, to confirm that IL-8 is able to induce keratocyte migration, we tested seven concentrations (0.25 ng/ml, 0.5 ng/ml, 0.75 ng/ml, 1 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml) of recombinant human IL-8 and performed scratch assay and chemotaxis assay. Cells were scratched with a 200-μl pipette tip and treated with various concentrations of IL-8. Images were taken at 0, 8, 24, and 48 hours to observe cell migration toward the center of the scratch. Cells were counted inside the created injury and compared with time 0. At 8 hours, all concentrations of IL-8 except 0.25 ng/ml and 100 ng/ml significantly increased keratocyte migration; 24 hours after scratch injury, all concentrations of IL-8 had a positive effect on cell migration. At 48 hours, only 0.5 ng/ml, 0.75 ng/ml, and 1 ng/ml of IL-8 significantly increased keratocyte migration (Fig. 6E). CytoSelect cell transwell migration assay was used to determine whether various concentrations of IL-8 are able to attract keratocytes. Keratocytes were allowed to migrate for 18 hours; 0.5 ng/ml, 0.75 ng/ml, and 1 ng/ml of IL-8 significantly enhanced keratocyte migration, whereas 0.25 ng/ml, 10 ng/ml, 50 ng/ml, and 100 ng/ml had a positive effect on the migration, although not statistically significant (Fig. 6F). Additionally, IL-8 had no effect on keratocyte proliferation (data not shown).

**SP Increases Neutrophil Migration Indirectly through IL-8.** To test whether IL-8 secreted by keratocytes after SP stimulation is able to attract neutrophils and/or whether SP is able to attract neutrophils directly, CytoSelect cell transwell migration assay was used. Isolated human neutrophils were used as migratory cells. Cells were allowed to migrate for 18 hours. First, supernatants collected from keratocytes untreated (control), treated with either SP (10^{-7} M), or pretreated with L-733,060 showed no formation of filopodia and lamellipodia nor focal adhesion points or stress fibers. Cells pretreated with wortmannin did not show actin reorganization (no formation of stress fibers, leading edge, filopodia, and lamellipodia) and did not express the focal adhesion protein (arrows), vinculin, at 8 and 48 hours (red). Cells treated with SP showed expression of focal adhesion (arrows) and lamellipodia (arrowheads) could be observed at the leading edge of migrating cells treated with SP at 8 and 48 hours (green). Prominent stress fibers were induced, especially at 48 hours (intracellularly; green). Cells treated with SP showed expression of focal adhesion protein (arrows), vinculin, at 8 and 48 hours (red). Cells pretreated with L-733,060 showed no formation of filopodia and lamellipodia nor focal adhesion points or stress fibers. Cells pretreated with wortmannin did not show actin reorganization (no formation of stress fibers, leading edge, filopodia, and lamellipodia) and did not express the focal adhesion protein (vinculin) either at 8 or at 48 hours.

![Image](http://www.molpharm.aspetjournals.org/attachment.php?attachmentid=123456)
and $10^{-5}$M or L-733,060 (10$^{-6}$M) and SP (10$^{-7}$ and 10$^{-5}$M), were used as chemoattractant. Concentrations of IL-8 were measured in these supernatants (Supplemental Table 4). The results showed that isolated human neutrophils migrated significantly more toward the supernatants collected from SP-treated keratocytes than toward control. Supernatants collected from L-733,060 and SP-treated cells did not enhance cell migration (Fig. 7A). Again, enhanced migration corresponded to higher concentrations of IL-8. Next, we tested whether SP is able to attract neutrophils directly. Fresh medium supplemented with two different concentrations of SP (10$^{-7}$ and 10$^{-5}$M) was used as a chemoattractant. Neither concentration of SP had a positive effect on isolated human neutrophils migration. Moreover, when neutrophils were pretreated with L-733,060 (10$^{-6}$M) and subsequently treated with SP, no migratory effect was observed (Fig. 7B), indicating that SP induced neutrophil migration through IL-8 but not directly.

**Discussion**

The cornea is the major refractive unit of the eye. Although relatively it is well protected from injury, when the cornea is injured, a proper wound-healing response in the cornea is needed to avoid scarring and subsequent visual loss or even blindness. SP and its receptor NK-1R have been identified in rat and rabbit corneal epithelial cells in which they are believed to play a role in maintaining corneal integrity and wound healing (Nakamura et al., 1997; 2003; Yamada et al., 2005). It has also been shown that human stromal keratocytes express SP and NK-1R (Watanabe et al., 2002), which has also been confirmed in our laboratory for the keratocyte cell culture model used in the present study (Sloniecka et al., 2015). Little is known, however, about the role of SP and its receptor in corneal stroma wound healing. In this study, we showed that injured cultured human keratocytes produce more SP than resting cells and that they are able to respond to SP stimulation with increased migration and with expression of the proinflammatory and chemotactic protein IL-8, which further stimulates the cells to migrate and also attracts neutrophils. Two different concentrations of SP were tested. The concentrations used were tested for toxicity and found not to be toxic to keratocytes. As no data on endogenous SP levels in vivo in the human cornea are available, it is not evident what concentrations should be used in in vitro studies. In other species such as, for example, the mouse and rabbit, the endogenous SP levels are 40.5–68.6 pg/cornea and 5.1 pmol/g, respectively (Keen et al., 1982; Stjernschantz et al., 1982). Data are available for SP levels in human tear fluid: 306 ± 96.5 pg/ml (Yamada et al., 2002). Compared with the concentrations of SP we have used in our experiments, the SP concentration in tear fluid is about 400 (for SP 10$^{-7}$M) and 40,000 (for SP 10$^{-5}$M) times less. This is reasonable, as SP in tear fluid would be much diluted compared with SP in the interstitial extracellular fluid around the keratocytes that produce and secrete SP. Moreover, the source of SP in the cornea in vivo is far more than keratocytes themselves, which makes it likely that the endogenous levels around keratocytes in vivo are far greater than what is produced in culture dishes in vitro. For instance, nerves, such as the trigeminal nerve, also produce SP (Garcia-Hirschfeld et al., 1994; Ko et al., 2014; Kowtharapu et al., 2014), and corneal epithelial cells also have been found to produce it (Watanabe et al., 2002). The choice of the two concentrations that have been used in this study is not intended to mimic the normal concentration in vivo but to test whether SP induces keratocyte migration in vitro.
based on other studies in which a wide range of SP concentrations have been used, including the two concentrations used in this study (Villablanca et al., 1994; Koon et al., 2004; Koon et al., 2007; Backman and Danielson, 2013; Yang et al., 2014).

Both concentrations enhanced keratocytes migration in a dose-dependent manner. Cell migration is initiated in response to various signals coming from cells or extracellular matrix (Ridley et al., 2003). Actin cytoskeleton reorganization, which includes formation of filopodia, lamellipodia, and stress fibers, are essential for successful cell motility (Lamalice et al., 2007). We found that treatment of keratocytes with SP leads to formation of filopodia and lamellipodia on the leading edge of the cells, and it increased actin stress fibers. Moreover, expression of focal adhesion protein, vinculin, was observed in the migrating cells. The cytoskeleton changes induced by SP lasted a long time, suggesting that the SP effect on cell migration is relatively persistent. Expression of Rac1 and RhoA (Ridley, 2001), two proteins involved in regulation of cytoskeleton, was increased in cells stimulated with SP. Their expression was diminished in cells for which the SP receptor was blocked, indicating that SP induced cell migration through its preferred receptor (NK1-R). PI3K is a major regulator of key cellular functions, such as cell growth, aging.

Fig. 5. SP upregulates IL-8 gene expression and protein secretion in keratocytes. (A) Stimulation of keratocytes with SP upregulated IL-8 gene expression in a dose-dependent manner with a peak at 6 hours, as quantified by RT-qPCR. (B and C) Western blot and densitometry analysis of keratocytes stimulated with SP (10^{-7}M and 10^{-5}M). SP stimulation resulted in phosphorylation of SAPK/JNK (46 kDa) with peaks at 1 and 5 minutes after stimulation and phosphorylation of NF-κB (65 kDa) with peaks at 15, 30, and 180 minutes after stimulation. SAPK/JNK (46 kDa) and NF-κB (65 kDa) served as unphosphorylated controls. β-actin was used as a loading control (45 kDa). (D) SP stimulation increased IL-8 secretion by keratocytes in a dose-dependent manner, as assessed by ELISA. (E) Pretreatment of cells with the NK-1R inhibitor L-733,060 significantly decreased the SP-enhanced IL-8 secretion, assessed 24 hours after treatment by ELISA. Values are means ± S.D. n.s., not significant. *P < 0.05; **P < 0.01; ***P < 0.001.
SP enhances keratocyte migration through PI3K, which also acts as an upstream activator of Rac1 and RhoA. By using Western blot analysis, we found that SP was not able to increase Rac1 and RhoA expression when PI3K was inhibited. Moreover, the changes in cytoskeleton induced by SP were no longer observed when cells were pre-treated with wortmannin. Taken together, our data suggest that SP-induced migration is mediated through IL-8. (A) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes. Cells were treated with various concentrations of IL-8. Cells were counted inside the created injury and compared with time 0 at 8, 24, and 48 hours after scratch. Treatment of cells with IL-8—neutralizing antibody significantly reduced SP-enhanced migration after 24 and 48 hours. Isotype control alone had no effect. (B) Quantification of transwell migration assay, in which a supernatant collected from keratocytes treated with either SP (10^{-5}M or 10^{-3}M) or L-733,060 (10^{-5}M) and SP, was used as a chemoattractant. Supernatant collected from cells treated with L-733,060 and SP had no effect on cell migration. (C) Quantification of transwell migration assay, in which a supernatant collected from keratocytes treated with SP (10^{-5}M) and/or with rabbit IgG control and/or with IL-8—neutralizing antibody, was used as a chemoattractant. Supernatants from cells treated with IL-8—neutralizing antibody and SP significantly reduced cell migration compared with supernatant from cells treated with SP alone. Isotype control had no effect. (D) Quantification of transwell migration assay, in which CXCR1 and CXCR2 receptors were blocked on keratocytes. Supernatant collected from keratocytes treated with SP (10^{-5}M) was used as a chemoattractant. Mouse IgG2A served as isotype control. Blocking CXCR1 and CXCR2 significantly reduced cell migration. (E) Quantification of keratocyte migration after scratch injury. Scratch injury was introduced to keratocytes. Cells were treated with various concentrations of IL-8. Cells were counted inside the created injury and compared with time 0 at 8, 24, and 48 hours after scratch. At 8 hours, all concentrations of IL-8, except 0.25 ng/ml and 100 ng/ml, significantly increased keratocyte migration. Twenty-four hours after scratch injury, all concentrations of IL-8 had a positive effect on cell migration. At 48 hours, only 0.5 ng/ml, 0.75 ng/ml, and 1 ng/ml of IL-8 significantly increased keratocyte migration. (F) Quantification of transwell migration assay, in which different concentrations of IL-8 were used as a chemoattractant. 0.5 ng/ml, 0.75 ng/ml, and 1 ng/ml of IL-8 significantly enhanced keratocyte migration. Values are means ± S.D. n.s., not significant. P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001.

and cell motility (Cain and Ridley, 2009), and it has been shown to activate Rac1 at the leading edge of the migrating cells (Welch et al., 2003). It is also involved in a SP signaling pathway leading to proinflammatory responses in mouse macrophages (Sun et al., 2009). In this study we showed that PI3K is critical for SP-induced cell migration by inhibiting PI3K with wortmannin. By using Western blot analysis, we found that SP was not able to increase Rac1 and RhoA expression when PI3K was inhibited. Moreover, the changes in cytoskeleton induced by SP were no longer observed when cells were pre-treated with wortmannin. Taken together, our data suggest that SP enhances keratocyte migration through PI3K, which also acts as an upstream activator of Rac1 and RhoA.

It has been reported that SP stimulates human colonic epithelial cells (Koon et al., 2005), human astrocytoma cells (Palma and Manzini, 1998), and human corneal epithelial cells (Tran et al., 2000) to produce the chemotactic protein IL-8. It has furthermore been shown that SP upregulates IL-8 gene expression in keratocytes, with a peak at 6 hours, and this increase involves phosphorylation of both SAPK/JNK and NF-kB, which controls the IL-8 expression at transcription level. (A) Supernatant from keratocytes with known concentrations of secreted IL-8 after either SP or L-733,060 and SP treatment was used as a chemoattractant. Neutrophils migrated significantly more when supernatant collected from SP-treated keratocytes was used. Supernatant collected from keratocytes treated with L-733,060 and SP had no effect on neutrophil migration. (B) SP was used as a direct chemoattractant in fresh medium. SP had no direct effect on neutrophil migration. Pretreating cells with L-733,060 and SP had no effect on neutrophil migration either. Values are means ± S.D. n.s., not significant. ***P < 0.001.
expression, it is not essential, whereas NF-κB is (Feoktistov et al., 1999). To have a significant increase in IL-8 expression, activation of NF-κB and at least one other pathway is needed (Hoffmann et al., 2002); however, maximal amounts of IL-8 can be achieved only if NF-κB pathway, SAPK/JNK pathway, and p38 MAPK are all activated (Hoffmann et al., 2002). The lack of p38 MAPK kinase activation after SP stimulation of keratocytes in the present study might explain the significant but not robust increase in IL-8 gene secretion and expression. SP-treated cells secreted more IL-8 protein than did controls, and this effect was dose dependent. Additionally, NK-1R was necessary to convey SP effect on IL-8 production. IL-8 is a chemoattractant, but it has also been shown to stimulate cell migration in endothelial cells (Lai et al., 2011) and cancer cells (Yin et al., 2014). Our results suggest that IL-8 secreted by keratocytes resulting from SP simulation is responsible for SP-enhanced keratocyte migration as established by the fact that neutralization IL-8 abolished the SP effect on keratocyte migration. Furthermore, using supernatants collected from keratocytes treated with SP and with measured IL-8 concentrations, we could see that increased keratocyte migration correlates to higher IL-8 concentrations in the supernatant. Interestingly, IL-8 neutralization revealed that endogenous levels of IL-8 produced by keratocytes made cells more motile since when control cells (not treated with SP) were treated with IL-8–neutralizing antibody, their migration was lower than control cells without IL-8 neutralization. Lastly, when IL-8 receptors CXCR1 and CXCR2 were blocked, migration of keratocytes was decreased, confirming that IL-8 is important in the SP-enhanced migration; however, other cytokines may also be produced by keratocytes as a result of SP stimulation and thereby contribute to the enhanced migration, as we also observed that recombinant IL-8 alone was not as effective in enhancing migration as SP or supernatants collected from SP treated cells. In fact, we found that at least two other cytokines are produced after SP treatment: monocyte chemoattractant protein-1; and regulated on activation, Normal T cell expressed and secreted (unpublished data).

The issue of the high level of basal IL-8 secretion in untreated keratocytes found in this study should be addressed. One possibility is that the cultured keratocytes are in an activated state owing to, for example, the presence of FBS in the culture. In a recent study of ours (Sloniecka et al., 2015), we examined the phenotype of the cells used in this study. As FBS is known to alter the phenotype of keratocytes and activate them, we compared keratocyte markers and activate them, we compared keratocyte markers in keratocytes grown with or without 2% FBS. Our results showed that the keratocytes grown both with and without FBS express keratocyte markers (such as keratocan, lumican, CD34, and ALDH) and express very low amounts of, for example, α-SMA (a marker of myofibroblasts), and therefore they are not in an activated state (Sloniecka et al., 2015). The reason for high basal IL-8 levels remains unknown; however, we observed that the basal IL-8 level is not passage dependent. It seems to be a cumulative event; that is, the longer the cells are in culture (at a specified passage), the more IL-8 is secreted. It has been shown that growing keratocytes in medium containing low glucose concentrations (1000 mg/liter) has been beneficial for maintaining keratocyte phenotype. Therefore, we tested whether medium containing low glucose concentration has an effect on IL-8 secretion by keratocytes (unpublished data). Our results have shown that there is a slight, but not significant, decrease in IL-8 secretion in keratocytes grown in the low glucose medium compared with the IL-8 secretion from cells grown in high glucose medium (3,151 mg/liter). We have also tested IL-8 secretion in keratocytes grown on collagen I (as the cornea is composed of collagen I). Surprisingly, keratocytes grown on collagen I secrete almost twice as much IL-8 compared with keratocytes grown on uncoated surface (both for high and low glucose media; unpublished data). No data are available to indicate how much IL-8 is produced by keratocytes or other cells in the healthy cornea in vivo; however, studies have shown that IL-8 in tear fluid in healthy individuals fluctuate between 148 and 414 pg/ml (Carreno et al., 2010).

Neutrophils play an important role in cornea protection. They provide protection against pathogens and foreign substances on the eye surface (Waring and Rodrigues, 1987). As the cornea has no blood supply or lymphatic drainage, however, the nearest source of neutrophils can be found in blood vessels of the corneal limbus (Van Buskirk, 1989), that is, in the periphery adjacent to the tunica conjunctiva. Therefore, a powerful chemokine is needed to attract neutrophils to the cornea, such as IL-8, which is known to be able to attract neutrophils into inflamed tissues (Baggilini et al., 1994). On the other hand, neutrophil infiltration in an injured cornea needs to be tightly controlled; otherwise, the release of various proteases by neutrophils may result in stromal degradation and ulceration, which in turn might lead to corneal opacity and neovascularization (Brown et al., 1970), causing decreased vision or even blindness. SP has been reported to chemoattract rabbit neutrophils (Marasco et al., 1981), activate human neutrophils (Serra et al., 1988), and increase neutrophil adhesion to bovine bronchial epithelial cells (DeRose et al., 1994); however, our results show that SP is not able to attract human neutrophils directly but that it does so by stimulating keratocytes to produce IL-8, which then attracts neutrophils.

In summary, we conclude that SP, binding to its preferred receptor NK-1R, through activation of IP3 kinases and the Rac1/RhoA pathway is able to enhance keratocyte migration after injury, thus facilitating wound healing. Moreover, SP stimulates keratocytes to produce IL-8, which further enhances cell migration and, as this IL-8 was also shown to be a chemoattractant of human neutrophils, it is possible that SP might help initiate acute inflammation within the corneal stroma after injury from trauma or infection.

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