The Role of Substance P in the Pathogenesis of Pterygia

Jeanie Chui, Nick Di Girolamo, Minas T. Coroneo, and Denis Wakefield

PURPOSE. Pterygium is a prevalent ocular surface disorder thought to be triggered by chronic ultraviolet damage to the limbus. One of the enigmatic features of pterygium is its wing-like shape, and the mechanism(s) supporting its centripetal growth remain to be elucidated. Because the growth pattern of pterygium mirrors the radial arrangement of corneal nerves, the authors propose that neuropeptides may facilitate its directional growth. This hypothesis prompted an investigation of the role of the sensory neuropeptide substance P (SP) and its receptor (NK₁ receptor) in directing cell migration in pterygia that may explain the characteristic growth pattern.

METHODS. Immunohistochemical analysis for SP and the NK₁ receptor was performed on five pterygium specimens with corresponding autologous conjunctiva and limbus. Migration of pterygium epithelium, fibroblasts, and vascular endothelial cells toward SP was assessed by using a modified Boyden chamber.

RESULTS. SP and NK₁ receptors were localized to infiltrating fibroblasts, mononuclear cells and the epithelia of pterygium, conjunctiva, and limbus, with elevated NK₁ receptor staining observed in pterygia. SP at nanomolar concentrations induced cell migration in pterygium fibroblasts and vascular endothelium in a dose-dependent fashion, which was inhibited by an NK₁ receptor antagonist. Pterygium epithelial cells were not migratory in these experiments.

CONCLUSIONS. For the first time, this study showed the presence of NK₁ receptor in pterygia and that SP is a potent chemoattractant for pterygium fibroblasts and vascular endothelial cells, implying that SP may contribute to the shape of pterygia through its profibrogenic and angiogenic action. (Invest Ophthalmol Vis Sci. 2007;48:4482–4489) DOI:10.1167/iovs.07-0123

Pterygium is a prevalent ocular surface disease thought to be triggered by excessive UVB exposure and damage to limbal stem cells¹ leading to aberrant wound-healing responses that are mediated by cytokines, growth factors, and matrix metalloproteinases.² Despite extensive research, certain unique aspects of pterygium pathogenesis remain unexplained. In particular, the mechanism for the centripetal growth pattern that gives rise to its wing-like appearance has not been determined. In an attempt to explain this phenomenon, we considered possible signals that govern centripetal migration in normal corneal epithelial cells as summarized by the XYZ hypothesis.³ In the past, several theories have been proposed to model centripetal migration, including population pressure from the peripheral cornea,⁴ preferential desquamation of central corneal epithelium,⁵ chemical signals from the stroma,⁶ and electrical cues.⁷ Adding to these theories, we hypothesized that corneal nerves may be involved, since the direction of epithelial cell movement follows the course of radial corneal nerves.⁸,⁹ Sensory corneal nerves express several neuropeptides, including substance P (SP), neurokinin A (NKA), calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating peptide (PACAP), and secretoneurin (SN).⁸,¹⁰,¹¹ Some of these may provide signals for cell migration, given that sensory denervation results in delayed corneal wound healing, recurrent erosions, and randomly arranged and poorly attached epithelial cells.¹²,¹³ Sympathetic denervation, however, caused increased epithelial thickness, edema, conjunctival hyperemia, stromal vascularization, enlarged corneal nerves, and periorbital pain.¹⁴,¹⁵ In addition, the focusing effect of the cornea, which concentrates UV light onto the limbus,¹ can injure corneal nerves as they leave the limbal plexus to enter the cornea proper. As sensory nerves are reported to participate in wound healing, inflammation, and cutaneous photo-aging,¹⁶,¹⁷ they could play similar roles in the UV-injured cornea and be responsible for directing cell migration in pterygia.

Sensory neuropeptides exert trophic influences on the normal cornea, but also play roles in inflammation and corneal wound healing.¹²,¹³,¹⁸,¹⁹ Therefore, we choose to investigate SP as a signal for cell migration in pterygium, given its reported roles in corneal cell migration, proliferation, and wound healing.¹⁹–²² SP belongs to the tachykinin family of peptides and is a product of the TAC1 gene that also encodes for NKA, neuropeptide K, and neuropeptide γ. Tachykinin receptors are members of the G protein-coupled receptor (GPCR) family and in mammals, NK₁, NK₂, and NK₃ have been identified where NK₁ is the preferred receptor to SP and hemokinin, whereas NK₂ and NK₃ preferentially bind to NKA and neurokinin B, respectively.²³ In the cornea, SP was first identified in nerves originating from the trigeminal ganglion.²⁴–²⁶ More recent studies also reported the presence of SP in corneal epithelium and keratocytes²⁷ and in normal tears.²⁸ SP exerts its trophic influences in synergy with other growth factors such as insulin-like growth factor (IGF)-I or epidermal growth factor (EGF),²¹,²² where the combination of IGF-I and SP was successfully used to treat corneal diseases such as neurotrophic keratopathy.²⁹–³⁰ Furthermore, the effects of SP may be potentiated by the presence of EGF in pterygia.³¹

The purpose of this study was to identify neuronal signals that could explain the wing-like growth and shape of pterygia. As there are no animal models of pterygia, we used primary cell cultures derived from a pterygium and control tissue to test whether the sensory neuropeptide SP can act as a signal for cell migration. The modified Boyden chamber cell migration assay was chosen to provide a gradient of chemoattractant as opposed to the organ-cultured corneal blocks favored by others,²¹,²² in which cell migration could be a function of proliferation and sliding movement of epithelium, similar to that which occurs during corneal wound healing. In our cell culture model, SP-induced concentration-dependent migration of pterygium fibroblasts (but not pterygium epithelium) and microvascular endothelium via the NK₁ receptor, implying that SP may function as a mediator of fibrosis and angiogenesis,

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processes that characterize the development of pterygium. Using immunohistochemical techniques, we localized the distribution of SP and NK\(_4\) receptors to resident fibroblasts, infiltrating mononuclear cells and basal pterygium epithelial cells. To our knowledge, this is the first description of the presence and functional significance of NK\(_4\) receptors in this disease.

**METHODS**

**Specimens**

Pterygia, limbus, and conjunctiva were obtained from patients undergoing pterygium excision surgery at Prince of Wales Hospital (Sydney, Australia). Institutional ethics committee approval and informed consent was obtained from each patient before tissue collection and complied with the tenets of the Declaration of Helsinki. Tissue was either used to establish primary cell cultures or was formalin fixed and paraffin embedded for histologic assessment.

**Cell Cultures**

Primary epithelial and fibroblast cultures were established by modifying a previously published method.\(^2\,3\) In brief, epithelium was stripped away from the body of the pterygium during surgery and cultured separately in Eagles minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamate, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin, in a six-well plate and left to attach. Epithelial cells migrated from explants between 3 and 5 days and displayed typical cobblestone morphology, as previously described.\(^3\) Fibroblasts migrated from the pterygium body at 7 to 14 days and were identified by their elongated spindle-shaped morphology. The purity of epithelial and fibroblast cultures were established using flow cytometry and epithelial markers, as previously described.\(^2\,3\)

An SV40-transformed human microvascular endothelial cell line (HMEC-1)\(^33\) was a gift from Levon M. Khachigian (Centre for Vascular Research, University of New South Wales [UNSW]). HMEC-1 cells were maintained in MCDB 131 medium supplemented with 10% FBS, 10 ng/mL epidermal growth factor, 1 \(\mu\)g/mL hydrocortisone, 2 mM L-glutamine, 5 \(\mu\)g/mL penicillin, and 5 \(\mu\)g/mL streptomycin and passaged twice weekly.

**Neuropeptides and Antagonists**

SP and a nonpeptide NK\(_4\) receptor antagonist L-732138 (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.5 M acetic acid and dimethyl sulfoxide (DMSO), respectively. Stock solutions (10\(^{-5}\)M) were aliquoted and stored at \(-20^\circ\)C.

**Cell Migration Assays**

Migration of pterygium epithelium, pterygium fibroblasts, and HMEC-1 toward SP was investigated by using a modified Boyden chamber method.\(^3\) Subconfluent cultures were harvested using 0.05% trypsin/0.02% EDTA solution, followed by several washes in PBS and binding medium (BM; consisting of 1% BSA in EMEM). Cells were left in BM to recover for 1 hour at 37\(^\circ\)C in a 5% CO\(_2\) incubator before use in migration assays.

A gradient of chemoattractant was set up between the lower wells of a 48-well chamber (Neuro Probe, Inc. Gaithersburg, MD) containing 26 \(\mu\)L of SP (10\(^{-10}\) to 10\(^{-7}\)M), and the upper wells containing cells in BM (2.5 \(\times\) 10\(^4\) in 50 \(\mu\)L per well). A polycarbonate filter (Neuro Probe, Inc.) with 10-\(\mu\)m pores was precoated with 0.5% gelatin in PBS and was used to separate the upper and lower wells. The cells were allowed to migrate toward the chemoattractant in a humidified incubator for 6 hours at 37\(^\circ\)C in the presence of 5% CO\(_2\). Cells that did not migrate were subsequently removed by gentle scraping of the upper side of the membrane, whereas the remaining cells were fixed in methanol and stained with Harris hematoxylin, and the filters were mounted on a glass slide. Cell migration was assessed by counting migrated cells in five high-powered fields (HPFs) per well at 400\(\times\) magnification with light microscopy (BH2 research microscope; Olympus Australia Pty. Ltd., Mount Waverly, Victoria, Australia). Cell migration toward a chemoattractant was normalized to that of BM alone and expressed as a chemotactic index (CI).

Receptor blockade experiments were performed as just described, with the exception that some cells were preincubated for 30 minutes with 0.1 to 10 nM of a nonpeptide antagonist specific to the NK\(_4\) receptor (L-732138)\(^3\) or vehicle (DMSO) before dispensing.

Checkerboard analyses were performed to differentiate between directional migration toward a chemotactic gradient (chemotaxis) and nondirectional or random cell migration (chemokinesis). The chemoattractant gradient that the cells were exposed to was altered by placing different concentrations of SP in both upper and lower wells of the chemotaxis chamber. The cells placed in the upper wells were allowed to migrate as just described.

**Immunohistochemical Analysis**

Immunohistochemical analysis was performed as previously described. Briefly, formalin-fixed, paraffin-embedded pterygia, limbus, and conjunctiva collected from patients undergoing pterygium excision surgery (\(n = 5\)) were cut (4 \(\mu\)m), dewaxed in xylene, and rehydrated through a graded series of ethanol. Antigen retrieval was performed by heating the sections in 10 mM sodium citrate (pH 6.0) for 10 minutes in a 750-W microwave oven (Panasonic, Osaka, Japan). Sections were incubated in 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase, washed three times in 1× Tris-buff ered saline (TBS; pH 7.6), then incubated for 20 minutes in 20% goat serum to block nonspecific binding. The sections were incubated in primary antibody (Table 1) overnight at 4\(\circ\)C, followed by several washes in TBS and were then incubated with either a biotinylated goat anti-rabbit or goat anti-mouse IgG (DakoCytomation, Glostrup, Denmark) at a 1:200 dilution for 30 minutes. Tissue sections were washed and incubated in 1:100 dilution of horseradish peroxidase conjugated-streptavidin (DakoCytomation) for 1 hour. Immunoreactivity was visualized by adding 3-amin-9-ethylcarbazole (AEC; Sigma-Aldrich). Sections were counterstained in hematoxylin and mounted in aqueous mounting medium (Crystal Mount; Biomedia Corp., Foster City, CA).

**Table 1.** Primary Antibodies Used for Immunohistochemistry and Flow Cytometry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Source</th>
<th>Clone</th>
<th>Catalog No.</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human tachykinin receptor 1</td>
<td>Rabbit</td>
<td>NovusBio</td>
<td>—</td>
<td>NLS1339</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-substance P</td>
<td>Rabbit</td>
<td>Penlabs</td>
<td>—</td>
<td>T-4107</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-human CD3</td>
<td>Rabbit</td>
<td>Dako</td>
<td>—</td>
<td>A0452</td>
<td>1:200</td>
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<tr>
<td>Anti-human CD20cy</td>
<td>Mouse</td>
<td>Dako</td>
<td>L26</td>
<td>M0755</td>
<td>1:1000</td>
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<tr>
<td>Anti-human CD68</td>
<td>Mouse</td>
<td>Dako</td>
<td>KPI4</td>
<td>M0814</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-human mast cell tryptase</td>
<td>Mouse</td>
<td>Dako</td>
<td>AA1</td>
<td>M7052</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse IgG1</td>
<td>Mouse</td>
<td>Dako</td>
<td>—</td>
<td>X0931</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit immunoglobulin</td>
<td>Rabbit</td>
<td>Dako</td>
<td>—</td>
<td>X0903</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

NovusBio (Novus Biologicals Inc., Littleton, CO); Penlabs (Peninsula Laboratories Inc., San Carlos, CA); Dako (DakoCytomation, Glostrup, Denmark).
Negative control reactions included sections incubated with an isotype antibody and sections incubated without primary antibody.

**Flow Cytometric Analysis of NK<sub>1</sub> Receptor Expression in Cell Cultures**

Trypsin digested cells were allowed to recover in complete medium at 37°C for 30 minutes, followed by fixation in 2% paraformaldehyde (2 minutes), and permeabilization in cold 100% methanol (10 minutes) and 0.1% Triton X-100/PBS (5 minutes). Cells were subsequently incubated with primary antibody (anti-tachykinin receptor 1 or control rabbit IgG at 1 μg/mL; Table 1), followed by the addition of a secondary biotinylated goat anti-rabbit IgG (DakoCytomation) at 1:200 dilution. Finally, 10 μL of streptavidin-PE (Sigma-Aldrich) was added to the cells. All incubations from primary antibody onward were performed on ice for 30 minutes each, and the cells were extensively washed in 2% BSA-PBS between each reagent. The cells were resuspended in 1% paraformaldehyde and analyzed with a flow cytometer (FACScan with CellQuest Pro software; BD Biosciences, San Jose, CA).

**Statistical Analysis**

Statistical analysis was performed with commercial software (Prism, ver. 4.00 for Windows; GraphPad Software, San Diego, CA). One-way or two-way ANOVA was used when appropriate.

**RESULTS**

**Effect of SP on Pterygium Fibroblast and Microvascular Endothelial Cell Migration**

SP dose dependently induced migration of pterygium fibroblasts (Fig. 1A) and microvascular endothelial cells (Fig. 1B) at concentrations ranging from 10<sup>-14</sup> to 10<sup>-6</sup> M with a maximum response at 10<sup>-8</sup> M for both cell types. Pterygium fibroblasts displayed a maximum CI ± SEM of 4.17 ± 0.12, and for vascular endothelial cells the peak CI was 2.53 ± 0.18. Limbal and pterygium-derived epithelial cells attached to the filters but did not migrate, even when the incubation time was extended from 6 hours to 24 hours (data not shown).

**Inhibition of SP-Induced Cell Migration in Pterygium Fibroblasts and Vascular Endothelial Cells by an NK<sub>1</sub> Receptor Antagonist**

SP-mediated pterygium fibroblast migration was inhibited by the presence of L-732138 at the concentrations tested (0.1–10 nM) compared with the vehicle control DMSO (Fig. 1C). SP-induced microvascular endothelial cell migration was inhibited only with equimolar concentration of NK<sub>1</sub> receptor antagonist at 10 nM (Fig. 1D). The expression of NK<sub>1</sub> receptors in cultured pterygium fibroblasts and microvascular endothelial cells was confirmed by flow cytometry (Fig. 2).

**Checkerboard Analysis of SP-Induced Cell Migration**

Since the observed cell migration toward SP could be a consequence of chemotaxis or chemokinesis, we performed checkerboard analysis for clarification. If a substance induces a purely chemotactic response, one would expect cell migration to occur only when a gradient is present, with little cell movement in its absence. Whereas, if a substance induces a chemokinetic response, there would be a general increase in...
random cell movement with increased concentration of the substance.

For pterygium fibroblasts, chemokinesis was demonstrated by an increase in the number of cells with increased concentration of SP, despite an absence of a gradient, as shown by the bold data on the diagonal in Table 2. SP also exerted a chemotactic effect as evidenced by increased cell migration with increased concentration of neuropeptide in the lower wells (top row, rightmost column), whereas little cell migration occurred with no chemoattractant in the lower wells (Table 2, leftmost column). Thus, SP exerts both chemokinetic and chemotactic effects on pterygium fibroblasts. Conversely, the effect of SP on HMEC-1 cells was predominantly chemokinetic, as evidenced by an increase in the number of cells with increased concentration of SP in the absence of a gradient (Table 3, bold data). Furthermore, cell migration occurred, irrespective of the direction of the gradient. This was illustrated in cells exposed to SP in the upper wells migrating to lower wells without SP (Table 3, leftmost column). These values were not significantly different to cells not exposed to SP in the upper wells migrating to lower wells with SP (Table 3, top row). Therefore, the chemotactic effect of SP on HMEC-1 is less than its chemokinetic effect.

**SP and NK₁ Receptor Immunoreactivity in Pterygia**

SP immunoreactivity was observed in pterygia and in the normal ocular surface, where it was localized to the cytoplasm of epithelial cells, keratocytes, pterygium fibroblasts, mononuclear, and vascular endothelial cells (Figs. 3A–E). In normal tissues, SP-immunoreactivity varied, from full-thickness staining in conjunctival epithelium (Fig. 3C) to basal staining in limbal and central corneal epithelium (Figs. 3D, 3E). At the pterygium head (Fig. 3A), basal epithelial SP immunoreactivity was noted and was similar to that observed in the limbus and normal cornea, whereas over the body of pterygia (Fig. 3B), SP staining in the epithelium was full thickness and similar to the conjunctiva. We did not observe any neuronal structures in any tissue sections.

Intense cytoplasmic NK₁ receptor immunoreactivity was observed in pterygium fibroblasts, infiltrating mononuclear cells and basal epithelial cells at the pterygium head with reduced but full-thickness staining in the epithelium covering the pterygium body (Figs. 3F, 3G). In contrast, very little NK₁ receptor expression was noted in the normal ocular surface (Figs. 3H–J), with the exception of NK₁ receptor positive mononuclear infiltrates present in some conjunctival sections (Fig. 3H). An unexpected finding was the absence of NK₁ receptor staining in intraepithelial capillaries and small blood vessels in pterygium and conjunctival specimens (Figs. 3G, 3H).

Clusters of NK₁ receptor–positive mononuclear cells were observed in subepithelial and in perivascular areas within the stroma of pterygia and conjunctival specimens (Figs. 3G, 3H). NK₁ receptor–positive cells typically had an indented and eccentrically located nucleus with prominent nucleolus and variable cytoplasm volume (Figs. 3G, 3H, 3K–N). NK₁ receptor–positive cells did not express CD3, CD20, CD68, or tryptase (Figs. 3K–R) when adjacent sections were stained for these markers, suggesting that they were not T- or B-lymphocytes, monocytes, macrophages, or mast cells, respectively.

**Table 2.** Checkerboard Analysis of SP-Induced Cell Migration in Pterygium Fibroblasts

<table>
<thead>
<tr>
<th>SP Concentration above the Filter (M)</th>
<th>0</th>
<th>10⁻¹⁶</th>
<th>10⁻¹²</th>
<th>10⁻⁸</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00 ± 0.12</td>
<td>1.63 ± 0.09</td>
<td>1.68 ± 0.19</td>
<td>1.95 ± 0.14</td>
</tr>
<tr>
<td>10⁻¹⁶</td>
<td>1.22 ± 0.16</td>
<td>1.44 ± 0.06</td>
<td>1.78 ± 0.13</td>
<td>1.68 ± 0.11</td>
</tr>
<tr>
<td>10⁻¹²</td>
<td>1.28 ± 0.16</td>
<td>2.48 ± 0.28</td>
<td>2.15 ± 0.09</td>
<td>3.02 ± 0.16</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>1.00 ± 0.16</td>
<td>1.61 ± 0.26</td>
<td>1.62 ± 0.11</td>
<td>2.45 ± 0.26</td>
</tr>
</tbody>
</table>

Data are expressed as chemotactic index ± SEM (n = 3).
Our observations, while adding to these findings, suggest that SP activation of NK₁ receptors in vascular endothelial cells, induced a predominately chemokinetic effect, that was partially suppressed with the NK₁ receptor antagonist L-732138. The lack of complete suppression in both fibroblast and endothelial cell migration could be explained by activation of other tachykinin receptors which also bind SP but at a lower affinity. 

Encouraging results were obtained by others using the SP analogue [D-Arg¹, D-Trp⁵,⁷,⁹, Leu¹¹]SP, which also antagonizes a broad range of GPCRs and is reported to inhibit tumor-associated angiogenesis and IL-8 induced corneal neovascularization. These results suggest that a nonspecific GPCR antagonist could be more useful as an antiangiogenic agent, given the possible presence of multiple GPCRs and redundant pathways.

SP failed to induce cell migration in pterygium epithelial cell cultures in our experimental conditions (results not shown). This observation could reflect a variation in NK₁ receptor expression as observed in normal corneal epithelium and keratoocytes, and our observations that NK₁ receptors are highly expressed in pterygium fibroblasts compared with pterygium epithelial cells in vivo supports our cell migration data (Figs. 1, 3F). Although epithelial cells expressed NK₁ receptors in vivo, their lack of migration in vitro may be explained by the rapid breakdown of SP by proteases or that additional factors required by epithelial cells to migrate, which may be supplied through epithelial-stromal interactions in vivo are lacking in our in vitro model.

Our immunohistochemical investigations showed SP immunoreactivity in the normal ocular surface and in pterygia. The absence of neuronal elements in our sections may be explained by tissue thickness. It is also possible that surgical specimens of pterygia do not include nerves deep in the corneal stroma. NK₁ receptor immunoreactivity, while present in small amounts in the normal ocular surface, was upregulated in pterygia, where it colocalized to regions with SP staining, with the exception of the vascular endothelium where NK₁ receptor staining was absent. NK₁ receptor upregulation in pterygia may result from stimulation by TNF-α, IL-1β, or SCF. Furthermore, in the pterygia we identified a population of NK₁ receptor-positive cells that did not express markers for T-lymphocytes (CD3), B-lymphocytes (CD20), monocytes or macrophages (CD68), or mast cells (tryptase), and we propose that at his population may represent relatively undifferentiated cells. The presence of NK₁ receptor-positive mononuclear cells in pterygium and autologous conjunctival specimens suggest that these cells may have traveled to the ocular surface from the blood, perhaps attracted by the presence of SP.

From our data, SP could attract fibroblasts and endothelial cells locally while pterygium fibroblasts arise from peripheral fibroadipose tissue posterior to Tenon’s capsule, and new vessel growth from the anterior conjunctival circulation. Alternatively, endothelial cell progenitors (EPCs) from the bone marrow may travel to the cornea where SP may act as a signal to mobilize these progenitors. This idea has merit, because under conditions of inflammation SP is elevated locally in tissues (including tears of patients with pterygia) and systemically in plasma, where it functions as a chemoattractant for immune cells. Although our observations support SP as a chemoattractant for a population of unidentified mononuclear cells (which could include EPCs), the absence of NK₁ receptor staining in the vasculature of pterygia, conflicts with our cell migration data. It is possible that NK₁ receptors became upregulated in vascular endothelium under cell culture conditions or through immortalization. Alternately, NK₁ receptors could become downregulated in vivo once vasculogenesis is complete.

In conclusion, we demonstrated the presence and upregulation of NK₁ receptors in pterygia and that SP could induce migration of pterygium fibroblasts and vascular endothelium, suggesting that the NK₁ receptor may be a useful
target for pharmacological intervention. Given the radial pattern of corneal innervation, we postulate that this in turn may contribute to the characteristic growth pattern observed in pterygia.

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