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 chemottractant 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 cells leading to aberrant wound-healing responses that are mediated by cytokines, growth factors, and matrix metalloproteinases. 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. In the past, several theories have been proposed to model centripetal migration, including population pressure from the peripheral cornea, preferential desquamation of central corneal epithelium, chemical signals from the stroma, and electrical cues. 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. 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). 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. In addition, the focusing effect of the cornea, which concentrates UV light onto the limbus, 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-ageing, 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. 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. 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. In the cornea, SP was first identified in nerves originating from the trigeminal ganglion. More recent studies also reported the presence of SP in corneal epithelium and keratocytes and in normal tears. SP exerts its trophic influences in synergy with other growth factors such as insulin-like growth factor (IGF)-I or epidermal growth factor (EGF), where the combination of IGF-I and SP was successfully used to treat corneal diseases such as neurotrophic keratopathy. Furthermore, the effects of SP may be potentiated by the presence of EGF in pterygia.

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 chemotactant as opposed to the organ-cultured corneal blocks favored by others, 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.
processes that characterize the development of pterygium. Using immunohistochemical techniques, we localized the distribution of SP and NK1 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 NK1 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. 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 μg/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. Fibroblasts migrated from the pterygium body at 7 to 14 days and were identified by their elongated spindle-shaped morphology.

**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 μm), dewaxed in xylene, and rehydrated through a graded series of ethanols. 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-buffered 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°C, followed by several washes in TBS and visualized with a 1:100 dilution of horseradish peroxidase-conjugated streptavidin (DakoCytomation) for 1 hour. Immunoreactivity was visualized by adding 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich). Sections were counterstained in hematoxylin and mounted in aqueous mounting medium (Crystal Mount; Biomedica 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>
</tr>
<tr>
<td>Anti-human CD20cy</td>
<td>Mouse</td>
<td>Dako</td>
<td>L26</td>
<td>M0755</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-human CD68</td>
<td>Mouse</td>
<td>Dako</td>
<td>KPI</td>
<td>M0814</td>
<td>1:200</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 1/9262 g/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

---

**FIGURE 1.** NK<sub>1</sub> receptor mediation of SP-induced cell migration in pterygium fibroblasts and vascular endothelium. Migration of pterygium fibroblasts (A) and microvascular endothelial cells (HMEC-1) (B) toward various concentrations of SP normalized to BM control and dose-dependent inhibition of SP-induced migration of pterygium fibroblasts (C) and microvascular endothelial cells (HMEC-1) (D) by the nonpeptide NK<sub>1</sub> receptor antagonist (L-732138) relative to vehicle control (DMSO). Data expressed as the mean chemotactic index ± SEM. (A, B; n = 3, *P < 0.05, †P < 0.01, one-way ANOVA, Dunnett posttest and C, D; n = 6, *P < 0.05, †P < 0.001, two-way ANOVA, Bonferroni posttest).
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 chemotactic and chemokinetic 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. 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 cytoplasmic 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 signal to mobilize these progenitors.58 This idea has merit, because under conditions of inflammation SP is elevated locally in tissues (including tears of patients with pterygia)58,59 and new vessel growth from the anterior conjunctival circulation.38 Alternatively, endothelial cell progenitors (EPCs) from the bone marrow may travel to the cornea where SP may act as a chemoattractant for immune cells.61 Although our observations support SP as a chemoattractant for a population of unidentified mononuclear cells,59 and systemic 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 where pterygium fibroblasts arise from periorbital fibroadipose tissue posterior to Tenon’s capsule,77 and new vessel growth from the anterior conjunctival circulation.38 Although endothelial cell progenitors (EPCs) from the bone marrow may travel to the cornea where SP may act as a signal to mobilize these progenitors.59 This idea has merit, because under conditions of inflammation SP is elevated locally in tissues (including tears of patients with pterygia)58,59 and systemic lymphocytes, 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.

In conclusion, we demonstrated the presence and up-regulation 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

### Discussion

The fibrovascular nature of pterygium is well described and is thought to be due to the action of several fibroangiogenic growth factors and cytokines.60–64 However, these molecules alone cannot explain the growth pattern of invading fibroblasts or blood vessels in pterygia. One area previously overlooked is the potential influence of corneal nerves and neuropeptides in determining centripetal migration. The normal cornea is densely innervated with approximately 7000 nociceptors per square millimeter,8 and studies have reported myelinated and unmyelinated nerves within the connective tissue mass of pterygia,42,45 where nerves potentially act as a scaffold for invading fibroblasts and blood vessels. This concept is supported by the association of capillary sprouts with deep stromal nerves44 and patients with pterygia have reduced corneal sensation45 suggestive of dysfunctional nerves, similar to sensory changes observed in perineural invasion by cutaneous malignancies.46

In this study, we found that SP is a potent chemoattractant for pterygium fibroblasts, active at nanomolar concentrations. Supporting our observations, SP is reported to induce migration and proliferation, and to upregulate the production of the fibrogenic growth factor TGF-β in skin fibroblasts.54–57 Furthermore, SP and the NK1 receptor are overexpressed during abdominal adhesion formation in an animal model of mice which is reversed with an NK1 receptor–specific antagonist,49 indicating that this process is open to pharmacologic intervention.

The fibroangiogenic effect of SP is implicated by its ability to mediate proliferation, migration, adhesion molecule expression, and formation of capillary tube-like structures in endothelial cell cultures.50–52 and in animal models58,59 and in animal models SP-induced neovascularization in corneas, SC tissues, and knee joints.50–52 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 our in vitro model.

### Table 3. Checkerboard Analysis of SP-Induced Cell Migration in Microvascular Endothelium

<table>
<thead>
<tr>
<th>SP Concentration above the Filter (M)</th>
<th>SP Concentration below the Filter (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>10^{-16}</td>
<td>1.50 ± 0.08</td>
</tr>
<tr>
<td>10^{-12}</td>
<td>1.47 ± 0.07</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>1.67 ± 0.10</td>
</tr>
</tbody>
</table>

Data expressed as chemotactic index ± SEM (n = 3).
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.

Acknowledgments

The authors thank Maria Sarris and Gavin Mackenzie from the Histology and Microscopy Unit, UNSW, for their assistance.

References

48. Reed KL, Fruin AB, Bishop-Bartolomei KK, et al. Neurokinin-1 receptor and substance P messenger RNA levels increase during


