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The NK_1 receptor and its participation in the synergistic enhancement of corneal epithelial migration by substance P and insulin-like growth factor-1

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1 We have previously shown that substance P (SP) and insulin-like growth factor-1 (IGF-1) act synergistically to enhance the migration of rabbit corneal epithelial cells in an organ culture model. The present study was designed to identify the epithelial cell SP receptor that participates in this synergistic effect.

2 Rabbit corneal blocks were incubated for 24 h, then the length of the path of epithelial migration was measured. Reagents tried in the TC-199 culture medium, in the presence or absence of IGF-1, were: SP, agonists of tachykinin receptors NK_1, NK_2 or NK_3 and antagonists of tachykinin receptors NK_1 or NK_2.

3 The binding characteristics of SP receptors were examined in rabbit cultured corneal epithelial cells by binding assays with [^{125}I]-SP in the presence or absence of excess unlabelled SP or ligands of NK_1, NK_2 or NK_3 receptors.

4 As was demonstrated previously, SP and IGF-1 stimulated epithelial migration when they were added to the culture medium together, but individually they had no effect. NK_1 agonists had the same synergistic effect with IGF-1 as did SP, but the NK_2 and NK_3 agonists did not. Furthermore, the NK_1 antagonist abolished the synergistic effect of SP and IGF-1, but the NK_2 antagonist had no effect.

5 SP bound specifically to rabbit cultured corneal epithelial cells. The binding affinity was 0.44 nM and there were 2.43×10^4 binding sites per cell. The NK_1 ligand competed, in a dose-dependent fashion, with the binding of SP to corneal epithelial cells, but neither the NK_2 nor NK_3 ligand affected binding.

6 We conclude that the SP receptor in rabbit corneal epithelial cells is NK_1 and that this receptor participates in the synergistic enhancement of corneal epithelial migration by SP and IGF-1. The precise mechanism(s) of this interaction requires more study. These findings imply that both neural and humoral factors are essential for the maintenance and healing of corneal epithelium.

Keywords: Substance P; insulin-like growth factor-1; tachykinin receptors; NK_1 receptor; epithelial migration; cornea

Introduction

Tachykinins, a class of neuropeptides, have a common C-terminal amino acid sequence, Phe-Xaa-Gly-Leu-Met-NH_2 (Dutta, 1993). The major mammalian tachykinins are substance P (SP), neurokinin A and neurokinin B. Their physiological functions are elicited by binding to three distinct receptors whose existence has been demonstrated by physiological, functional tests, protein sequencing and cDNA cloning. These tachykinin receptors, designated NK_1, NK_2 and NK_3, show preferred affinity for SP, neurokinin A and neurokinin B, respectively (Lavielle *et al*., 1988; Regoli *et al*., 1988). Tachykinins mediate the various physiological functions that characterize neurogenic inflammation and wound healing (Pernow, 1983; Payan, 1989; Sterrett, 1990). At inflammatory sites, the NK_1 receptor has been shown to mediate physiological functions of SP such as fibroblast proliferation, plasma leakage and cellular migration (Ziche *et al*., 1990; Abelli *et al*., 1991; Kahler *et al*., 1993a, b).

SP, a constituent of sensory nerve fibres, is thought to be a neurotransmitter mediating functions such as neurogenic inflammation and the transmission of pain in various tissues (Payan, 1989; Otsuka & Yoshioka, 1993). It has been found to play an important role in ocular neurogenic responses to various stimuli (Unger *et al*., 1981; Nishiyama *et al*., 1981; Beding-Barnekow *et al*., 1988). In the cornea, one of the most heavily innervated and sensitive structures in the body, dense networks of SP-positive nerve fibres have been demonstrated by immunohistochemical techniques (Miller *et al*., 1981; Tervo *et al*., 1981). However, the specific role of SP in corneal physiology is not yet fully understood.

Reid *et al*. (1993) recently found that SP stimulated DNA synthesis in established corneal epithelial cells from the rabbit; treatment with SP made the cells more responsive to the subsequent addition of a secondary hormone, such as insulin. By use of a corneal organ culture model, we found that SP and insulin-like growth factor-1 (IGF-1) failed to affect corneal epithelial migration individually, but in combination they stimulated epithelial migration synergistically, in a dose-dependent fashion (Nishida *et al*., 1997).

The present studies were designed to elucidate the synergistic effect of SP and IGF-1 on corneal epithelial migration. In order to establish whether NK_1 is, as other studies imply (Lavielle *et al*., 1988; Regoli *et al*., 1988; Abelli *et al*., 1991), the SP receptor that participates in this effect, we used the organ culture model with medium containing SP and an agonist or antagonist of NK_1, NK_2 or NK_3 in the presence or absence of IGF-1. We also examined SP receptors in rabbit cultured corneal epithelial cells by conducting binding assays with [^{125}I]-SP in the presence or absence of excess unlabelled SP or ligands of NK_1, NK_2 and NK_3 receptors.

Methods

Epithelial migration

The length of the path of the epithelial migration over the cut stromal surface of a block of rabbit cultured cornea was

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measured as described previously (Nishida *et al*., 1983; 1990). In brief, full-thickness corneal blocks (approximately 2×4 mm) were cut with a razor blade from excised corneas and placed in each well of a 24-well tissue culture dish with unsupplemented TC-199 culture medium (control) or TC-199 containing the agents to be examined. SP, the NK_1 agonist (GR73632) and NK_1 antagonist (Sendide) were each tried at concentrations of 0.5, 1, or 2×10^{-5} M; the NK_2 agonist ([β-Ala^8] neurokinin A (4–10)), NK_2 antagonist (GR83074) and NK_3 agonist ([Pro^7] neurokinin B) were each tried at 2×10^{-5} M; IGF-1 10 ng ml^{-1} was used. After incubation for 24 h at 37°C under humidified 5% CO_2 in air, the specimens were fixed with a mixture of glacial acetic acid and absolute ethanol (5 : 95 v/v) at 4°C overnight. They were then dehydrated through graded ethanol, immersed in xylene and embedded in paraffin. Thin sections (4 μm) were cut, deparaffinized and stained with hematoxylin-eosin. Specimens were observed under a light microscope; photographs were taken and the length of the path of the corneal epithelium was measured on the printed photographs. Data are expressed as mean ± s.e.mean of 6 determinations.

Binding assay for SP

Corneal epithelial cells were prepared and cultured as described previously (Nishida *et al*., 1988). In brief, the endothelial layers of excised rabbit corneas were removed mechanically and the remaining corneal tissue was incubated with dispase (2 mg ml^{-1} in TC-199) for 1 h. The epithelial sheets were harvested and further incubated with trypsin (0.125%) and EDTA (0.01%) to make single cell suspensions. The cells were cultured on 24-well culture plates (5×10^4 cells/well) in rabbit corneal growth medium (RCGM) until they reached confluence. The culture medium was then changed to TC-199 containing 1% FBS (low-serum medium) and culture was continued for another 24 h.

The cells were washed once with 1 ml of assay buffer (TC-199 containing 0.1% BSA, 50 μg ml^{-1} chymostatin, 20 mM HEPES). Triplicate samples were incubated at room temperature for 1 h in 300 μl of assay buffer with [^{125}I]-SP (0.156, 0.313, 0.625, 1.25 or 5 nM) or, to determine nonspecific binding, with [^{125}I]-SP and 100 fold excess of unlabelled SP. The medium was removed; the cells were washed three times with ice-cold PBS and solubilized in 500 μl of 1 N NaOH. Radioactivity was measured with a gamma scintillation counter (COBRA II, Parkard, Meriden, CT). Specific binding was calculated by subtracting the non-specific binding from total binding. The cells in control wells were trypsinized and counted to determine the total number of cells per well. The binding affinity and number of binding sites for SP were determined by Scatchard plot analysis.

For competitive inhibition studies, cells were incubated with 1.25 nM [^{125}I]-SP and various concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M) of unlabelled SP or ligands of NK_1 (GR73632), NK_2 ([Lys^5, MeLeu^9, Nle^{10}] neurokinin A (4–10)) or NK_3 ([Pro^7] neurokinin B). The cells were processed as described above. For studies determining the effect of IGF-1 on the binding affinity and number of binding sites for SP, the cells were incubated with 10 ng ml^{-1} of IGF-1 in TC-199 containing 1% FBS for 24 h before the binding assay for SP. Data are expressed as mean ± s.e.mean in triplicate samples.

Materials

Albino rabbits weighing 2 to 3 kg were obtained from Kitayama Labes (Kyoto, Japan). Care and treatment of animals adhered to the ARVO Resolution on the Use of Animals in Research. TC-199 culture medium, trypsin (0.25%) and EDTA (0.02%) were from the Research Foundation for Microbial Diseases of Osaka University (Suita, Osaka, Japan); foetal bovine serum (FBS) was from Flow Laboratories (North Ryde, Australia); rabbit corneal growth medium (RCGM) was from Kurabo (Neyagawa, Osaka, Japan); dispase was from Godo Shusei (Tokyo, Japan); HEPES was from Gibco (Grand Island, NY); plastic multi-well culture dishes were from Costar (Cambridge, MA). SP, bovine serum albumin fraction V (BSA) and chymostatin were from Sigma (St. Louis, MO). IGF-1 was from Becton Dickinson Labware (Bedford, MA). [^{125}I]-SP (1.85 MBq) was from Amersham Japan (Tokyo, Japan). The remaining reagents were from Neosystem Laboratories, Strasbourg, France: NK_1 agonist was δ-aminovaleryl-[Pro^9, MeLeu^{10}] substance P (7–11) (GR73632) (Hagan *et al*., 1989); NK_1 antagonist was [Tyr^6, D-Phe^7, D-His^9] substance P (6–11) (Sendide) (Sakurada *et al*., 1992); NK_2 agonist was [β-Ala^8] neurokinin A (4–10) (Evangelista *et al*., 1990); NK_2 antagonist was Boc-Arg-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2 (GR83074) (McElroy *et al*., 1992); NK_3 agonist was [Pro^7] neurokinin B (Lavielle *et al*., 1988a, b) and the NK_2 ligand used in the SP binding assay was [Lys^5, MeLeu^9, Nle^{10}] neurokinin A (4–10) (Chassain *et al*., 1991).

Statistical analysis

Statistical analysis was carried out by an unpaired Student's *t* test for comparison of 2 groups, and by the Dunnett's multiple comparison test for comparison of 3 or more groups.

Results

First we confirmed the synergistic effect of SP and IGF-1 on corneal epithelial migration. Corneal blocks were cultured with SP at various concentrations (0.5, 1 or 2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}) for 24 h (Figure 1). SP did not affect the length of the path of epithelial migration in the absence of IGF-1. However, in the presence of IGF-1, epithelial migration increased in proportion to the concentration of SP. At SP concentrations of 1 or 2×10^{-5} M, epithelial migration was increased significantly, compared with unsupplemented cultures ($P < 0.01$).

We then attempted to determine which subtype of tachykinin receptor participates in this synergistic effect of SP and IGF-1. First we examined the NK_1 agonist (GF73632) (Figure 2). When corneal blocks were cultured in the presence of the NK_1 agonist alone, the length of the path of corneal epithelium was not affected. However, in the presence of IGF-1, the NK_1 agonist stimulated corneal epithelial migration in a dose-dependent fashion. At the higher concentrations of the NK_1 agonist (1 or 2×10^{-5} M), epithelial migration was increased

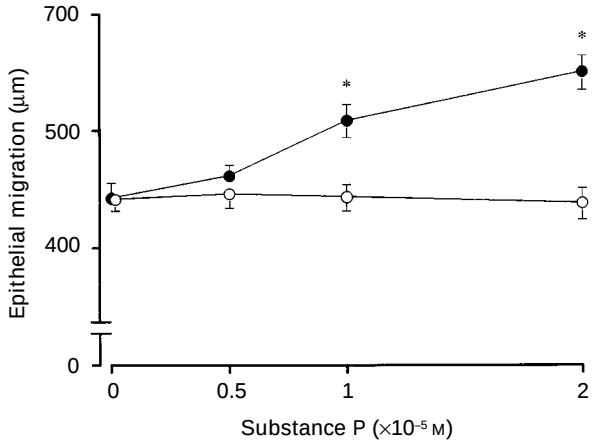

Figure 1 Synergistic effect of substance P (SP) and insulin-like growth factor-1 (IGF-1) on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing SP (0.5, 1 or 2×10^{-5} M) in the absence (○) or presence (●) of IGF-1 (10 ng ml^{-1}). Vertical lines represent s.e.mean of 6 determinations. *$P < 0.01$, compared with cultures in unsupplemented TC-199.

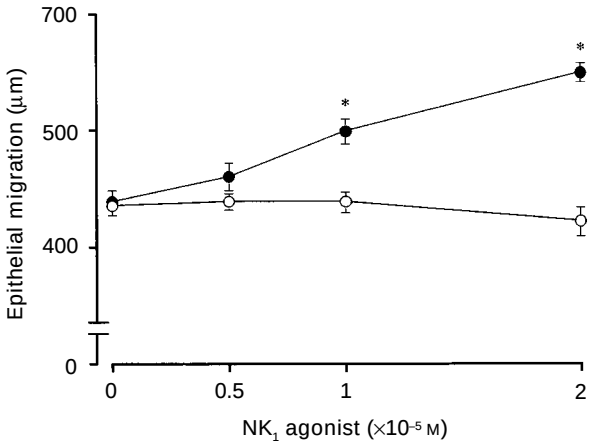

Figure 2 Effect of the NK_1 agonist, GR73632, on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing the NK_1 agonist (0.5, 1 or 2×10^{-5} M) in the absence (○) or presence (●) of insulin-like growth factor-1 (IGF-1; 10 ng ml^{-1}). Vertical lines represent the s.e.mean of 6 determinations. *$P<0.01$, compared with cultures in unsupplemented TC-199.

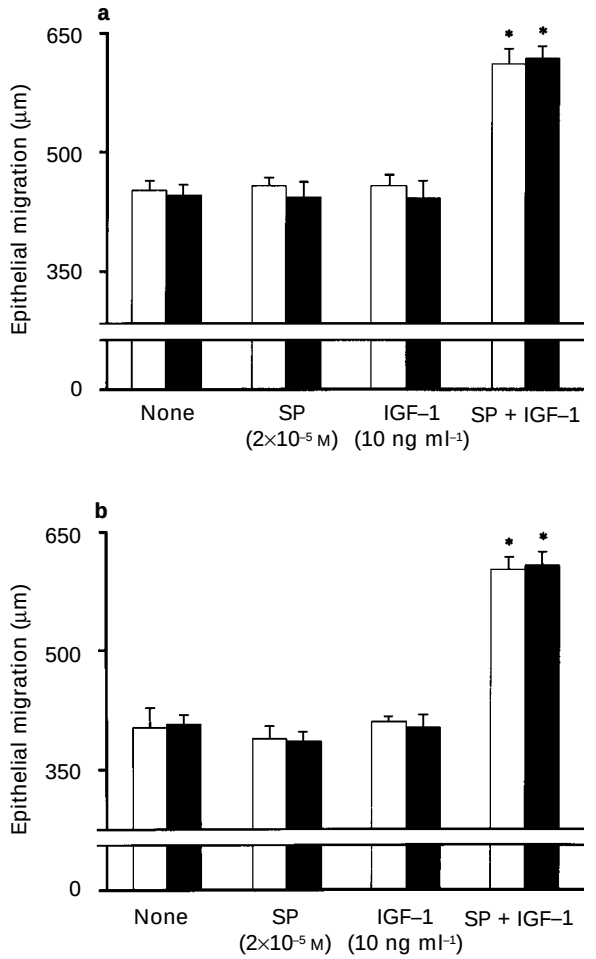

Figure 3 (a) Effect of the NK_2 agonist, [β-Ala^8] neurokinin A (4–10), on corneal epithelial migration. (b) Effect of the NK_3 agonist, [Pro^7] neurokinin B, on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing substance P (SP, 2×10^{-5} M), insulin-like growth factor-1 (IGF-1, 10 ng ml^{-1}) or a combination of SP and IGF-1 in the absence (open columns) or presence (solid columns) of neurokinin agonist (2×10^{-5} M). Each column represent the mean ± s.e.mean of 6 determinations. *$P<0.01$, compared with epithelial migration in unsupplemented TC-199.

significantly, compared with cultures in unsupplemented TC-199 ($P<0.01$). Thus, the NK_1 agonist showed the same synergistic effect with IGF-1 as did SP.

Neither the NK_2 agonist nor the NK_3 agonist, each at 2×10^{-5} M, added alone, with SP, with IGF-1, or with a combination of SP and IGF-1, affected corneal epithelial migration (Figure 3).

We also examined the effect of the NK_1 antagonist (Sendide, at 0.5, 1 or 2×10^{-5} M) on corneal epithelial migration. The NK_1 antagonist did not affect corneal epithelial migration alone or in cultures containing either SP or IGF-1. However, when both SP and IGF-1 were present, the NK_1 antagonist diminished their synergistic effect on epithelial migration in a dose-dependent manner. At 2×10^{-5} M, the NK_1 antagonist completely abolished the synergistic effect of SP and IGF-1 (Figure 4).

The NK_2 antagonist (GR83074) had no effect on corneal epithelial migration, regardless of whether it was added to the culture medium by itself, with SP, with IGF-1, or with SP and IGF-1 combined (Figure 5).

We then used a binding assay to learn more about the SP receptors of corneal epithelial cells. The specific binding of [^{125}I]-SP to its receptor on the corneal epithelial cells was saturated at concentrations of free radioactive ligand above 3×10^6 c.p.m. (Figure 6). Scatchard analysis revealed a single-component binding curve that corresponded to $2.43\pm0.08\times10^4$ binding sites per cell with a K_D of 0.441 ± 0.018 nM (Figure 7). Competitive inhibition studies (Figure 8) showed that unlabelled SP and the NK_1 ligand inhibited the binding of [^{125}I]-SP to cultured corneal epithelial cells in a dose-dependent fashion. Neither the NK_2 ligand nor the NK_3 ligand affected the binding of [^{125}I]-SP.

Finally, to investigate possible mechanisms of the synergy between SP and IGF-1, we attempted to determine whether IGF-1 modified the cells' binding affinity and/or number of binding sites for SP (Figure 9). Corneal epithelial cells were cultured in the absence or presence of IGF-1 for 24 h and submitted to the SP binding assay. Regardless of whether the cells were cultured in the presence of IGF-1 or not, the binding affinities and numbers of binding sites for SP were similar (Table 1). Scatchard analysis revealed identical single-component binding curves.

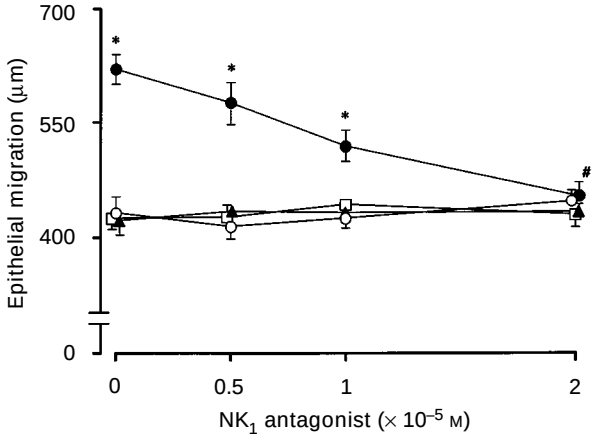

Figure 4 Effect of the NK_1 antagonist, sendide, on the synergistic effect of substance P (SP) and insulin-like growth factor-1 (IGF-1). Corneal blocks were cultured for 24 h in TC-199 containing the NK_1 antagonist (0.5, 1 or 2×10^{-5} M) in the absence or presence of SP (2×10^{-5} M) and/or IGF-1 (10 ng ml^{-1}). (○) Cultures contained the NK_1 antagonist alone; (▲) the NK_1 antagonist and SP; (□) the NK_1 antagonist and IGF-1. (●) The NK_1 antagonist, SP and IGF-1. Vertical lines represent s.e.mean of 6 determinations. *$P<0.01$, compared with cultures in unsupplemented TC-199. #$P<0.01$, compared with cultures containing both SP and IGF-1.

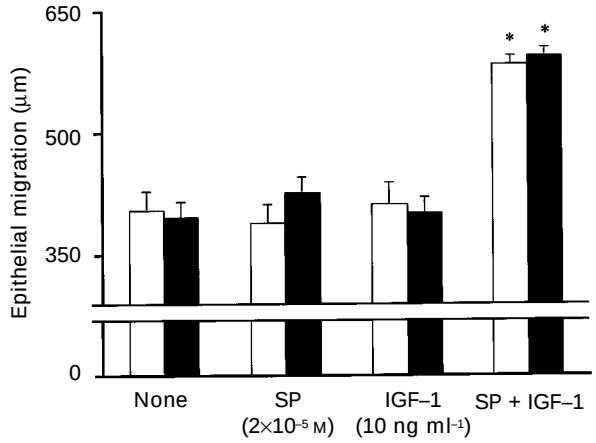

Figure 5 Effect of the NK_2 antagonist GR83074 on corneal epithelial migration. Cultures contained substance P (SP, 2×10^{-5} M), insulin-like growth factor-1 (IGF-1, 10 ng ml^{-1}) or a combination of SP and IGF-1 in the absence (open columns) or presence (solid columns) of NK_2 antagonist (2×10^{-5} M). Each column represents the mean ± s.e.mean of 6 determinations. *$P<0.01$, compared with unsupplemented control culture.

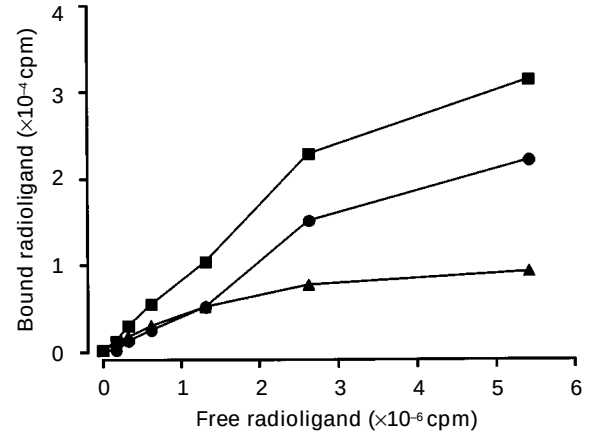

Figure 6 Saturation curve of [^{125}I]-substance P (SP) binding to cultured corneal epithelial cells. Cells were incubated with [^{125}I]-SP (0.156, 0.313, 0.625, 1.25 or 5 nM) in assay buffer at room temperature for 1 h. The cells were solubilized and total binding (■) was determined with a gamma scintillation counter. Non-specific binding (●) was determined by incubating cells with [^{125}I]-SP and a 100 fold excess of unlabelled SP. Specific binding (▲) was calculated by subtracting the non-specific binding from total binding. These experiments were performed in triplicate.

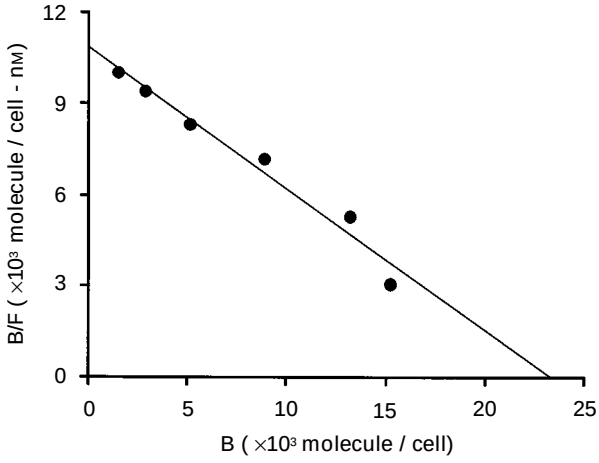

Figure 7 Scatchard plot of [^{125}I]-substance P (SP) binding to cultured corneal epithelial cells. Data were the analysed results of Figure 6 and best fitting lines determined by linear regression.

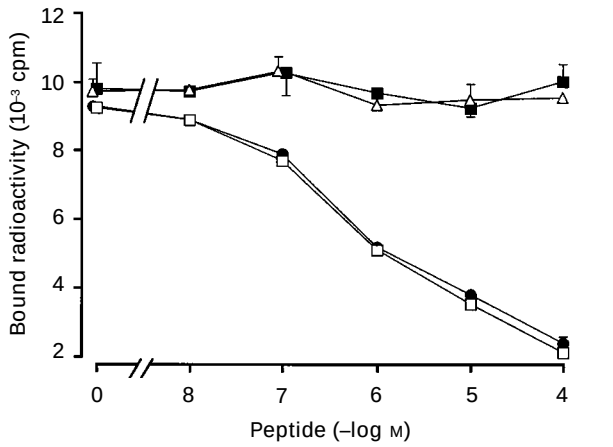

Figure 8 Competitive inhibition of [^{125}I]-substance P (SP) binding by unlabelled SP or ligands of NK_1, NK_2 or NK_3. Cultured corneal epithelial cells were incubated with 1.25 nM [^{125}I]-SP and various concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M) of unlabelled SP (□) or ligands of NK_1 (GR73632, ●), NK_2 ([Lys^5, MeLeu^9, Nle^{10}] neurokinin A (4–10), △) or NK_3 ([Pro^7] neurokinin B, ■) for 1 h at room temperature. The cells were solubilized and radioactivity was measured with a gamma scintillation counter.

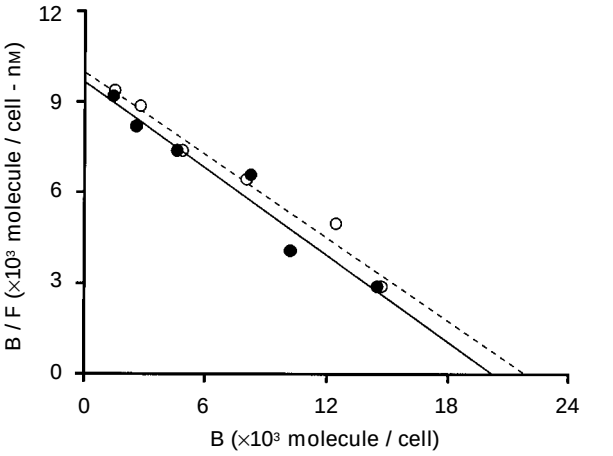

Figure 9 Scatchard plot analysis of the effect of insulin-like growth factor-1 (IGF-1) on the binding affinity and number of binding sites for substance P (SP) in cultured corneal epithelial cells. The cells were cultured in TC-199 containing 1% FBS in the absence (●) or presence (○) of IGF-1 (10 ng ml^{-1}) for 24 h and submitted to binding assay in triplicate samples.

Discussion

Intact corneal innervation is important in the maintenance of normal corneal integrity; when corneal innervation is damaged by pathological conditions, morphological changes occur in the corneal epithelium and epithelial wound healing is delayed (Gilbard & Rossi, 1990; Baker *et al*., 1993; Araki *et al*., 1994). Therefore, factors released from sensory nerves appear to play an important role in corneal epithelial wound healing. Several humoral factors, such as growth factors and cytokines, also participate in corneal epithelial wound healing (Nishida, 1993; Dua *et al*., 1994; Gipson & Inatomi, 1995). These findings imply that interacting neural and humoral factors are essential for the maintenance and healing of the corneal epithelium.

We recently demonstrated that SP and IGF-1 do not influence the migration of corneal epithelium individually, but that they act synergistically to stimulate epithelial migration (Nishida *et al*., 1996). We hypothesized that this synergistic effect of SP and IGF-1 might be mediated by the NK_1 receptor on corneal epithelial cells. The present results demonstrate that

Table 1 Effect of insulin-like growth factor-1 (IGF-1) on the binding affinity and number of binding sites for substance P (SP) in cultured corneal epithelial cells

Treatment	*Binding affinity* (nM)	*Number of binding sites* ($\times 10^4$ binding sites/cell)
None	0.450 ± 0.015	2.13 ± 0.78
IGF-1 (10 ng ml^{-1})	0.446 ± 0.008	2.15 ± 0.65

The corneal epithelial cells were cultured in TC-199 containing 1% FBS in the presence or absence of IGF-1 (10 ng ml^{-1}) for 24 h and then subjected to a binding assay. Data are expressed as mean $\pm$ s.e.mean in triplicate samples.

SP receptors are present on rabbit cultured corneal epithelial cells and that the NK_1 tachykinin receptor is the SP receptor that mediates the synergistic effect of SP and IGF-1 on corneal epithelial migration. The NK_1 agonist produced the same synergistic effect with IGF-1 as did SP, and an NK_1 antagonist abolished the synergistic effect of SP and IGF-1.

Although dense networks of SP-positive nerve fibres and SP binding sites have been shown in the corneal epithelium (Miller *et al*., 1981; Tervo *et al*., 1981; Kieselbach *et al*., 1990; Denis *et al*., 1991), no studies, to our knowledge, have characterized the SP receptors of corneal epithelial cells. The present results demonstrated that cultured corneal epithelial cells have an SP binding ability and that the subtype of tachykinin receptor responsible for SP activity is NK_1. Scatchard analysis revealed a single-component binding curve, a binding affinity of 0.44 nM and 2.43×10^4 SP binding sites per cell. These findings were similar to results obtained in studies of NK_1 receptor in brain and other tissues. The NK_1 receptor in corneal epithelial cells revealed one class of high-affinity binding sites with K_D values of about 0.5 nM (Helke *et al*., 1990; Krause *et al*., 1992).

SP is the most potent ligand for the NK_1 receptor; neurokinin A and neurokinin B have 100–1000 times lower affinity for the NK_1 receptor than does SP (Helke *et al*., 1990; Krause *et al*., 1992). SP exhibits cross-reactivity with other subtypes of tachykinin receptors (Yokota *et al*., 1989; Shigemoto *et al*., 1990), probably because these receptors recognize the common C-terminal amino acid sequence of tachykinins (Ingi *et al*., 1991; Cascieri *et al*., 1992). However, our studies demonstrated that the NK_1 ligand successfully competed with SP binding to corneal epithelial cells in culture, but the NK_2 and NK_3 ligands did not. These results show that SP does not utilize NK_2 or NK_3 receptors in the stimulation of corneal epithelial migration.

The mechanisms of the synergistic effect of SP and IGF-1 on corneal epithelial migration are not clear. IGF-1 has recently been shown to activate the expression of fibronectin receptors in lens epithelial cells (Palmade *et al*., 1994). Therefore, it seemed possible that IGF-1 might up regulate SP receptors on the surface of corneal epithelial cells. We tested this possibility by measuring the effect of IGF-1 on the binding affinity and number of binding sites for SP in cultured corneal epithelial cells. Our results did not support the hypothesis; treatment of the cells with IGF-1 failed to influence the binding affinity and number of binding sites for SP significantly. Another possible mechanism is cross-talk of the intracellular signal transduction systems of SP and IGF-1. The intracellular signal transduction systems of SP and IGF-1 are the G-protein-mediated pathway and the tyrosine kinase pathway, respectively (Nakanishi, 1991; Blount & Krause, 1993; Jones & Clemmons, 1995; LeRoith *et al*., 1995). Recent studies focusing on cellular functions have demonstrated cross-talk of signal transduction systems, especially the G-protein-mediated and tyrosine kinase pathways (van Biesen *et al*., 1995; Lev *et al*., 1995; Moxham & Malbon, 1996). Clearly, further studies on the mechanisms of the synergistic effect of SP and IGF-1 are needed.

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