Synergistic effect with Phe-Gly-Leu-Met-NH$_2$ of the C-terminal of substance P and insulin-like growth factor-1 on epithelial wound healing of rabbit cornea

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1   We previously reported that substance P and insulin-like growth factor-1 (IGF-1) synergistically stimulate corneal epithelial wound healing *in vitro* and *in vivo*. We wished to identify which portion of the amino acid sequence of substance P might be responsible for this synergism.

2   Corneal epithelial migration was not affected by the addition of any one of the following factors: substance P; Phe-Gly-Leu-Met-NH$_2$ (C-terminal of substance P); Val-Gly-Leu-Met-NH$_2$ (C-terminal of neurokinin A, neurokinin B, and kassinin); Tyr-Gly-Leu-Met-NH$_2$ (C-terminal of physalaemin); Ile-Gly-Leu-Met-NH$_2$ (C-terminal of eledoisin); or Gly-Leu-Met-NH$_2$ (common C-terminal of tachykinins).

3   In the presence of IGF-1, only substance P and Phe-Gly-Leu-Met-NH$_2$ were synergistic in stimulating corneal epithelial migration in a dose-dependent fashion.

4   The combination of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 did not affect the incorporation of [^{3}H]-thymidine into corneal epithelial cells.

5   Treatment with Phe-Gly-Leu-Met-NH$_2$ and IGF-1, but not with Phe-Gly-Leu-Met-NH$_2$ or IGF-1 alone, increased attachment of corneal epithelial cells to a fibronectin matrix.

6   The levels of α5 and β1 integrin were not affected by Phe-Gly-Leu-Met-NH$_2$ or IGF-1 alone, but they were significantly increased by the combination of Phe-Gly-Leu-Met-NH$_2$ and IGF-1.

7   Topical application of the same combination facilitated corneal epithelial wound closure *in vivo*.

8   These results demonstrated that Phe-Gly-Leu-Met-NH$_2$, a sequence of 4 amino-acids of the C-terminal of substance P, is the minimum sequence necessary to produce the synergistic effects of substance P and IGF-1 on corneal epithelial wound healing.

Keywords: Substance P; insulin-like growth factor-1; wound healing; epithelial migration; cell attachment; integrin

Abbreviations: BSA, bovine serum albumin fraction V; EGF, epidermal growth factor; FBS, foetal bovine serum; GLM, Gly-Leu-Met-NH$_2$; IGLM, Ile-Gly-Leu-Met-NH$_2$; IGF-1, insulin-like growth factor-1; FGLM, Phe-Gly-Leu-Met-NH$_2$; RT–PCR, reverse transcription-polymerase chain reaction; SP, substance P; YGLM, Tyr-Gly-Leu-Met-NH$_2$; VGLM, Val-Gly-Leu-Met-NH$_2$

Introduction

The epithelium is an important barrier that protects and maintains the structure and functions of the inside of the body against a variable external environment. Because the epithelium is often injured, however, it requires a speedy healing process. Various biological active substances, such as growth factors and extracellular matrix proteins, have been reported to modulate and regulate epithelial wound healing (Nishida, 1993; Woodley, 1996). However, the role of neural factors in epithelial wound healing is not yet fully understood. Clinically delayed wound healing has been noted in patients who have lost sensation through diseases such as diabetes mellitus.

The cornea is more heavily innervated with sensory nerve fibres than any other tissue in the body, and corneal innervatioan also plays an important role in the maintenance of corneal structure and functions. When corneal nerves from a branch of the trigeminal nerve are damaged by injury or disease, a variety of abnormalities result. In experimental studies, corneal denervation has been reported to result in

epithelial changes: increased permeability, decreased proliferation, changed phenotype, and delayed wound healing (Mishima, 1957; Baker *et al.*, 1993; Araki *et al.*, 1994). In the clinical setting, after various types of corneal injuries or diseases, such as trauma, intraocular surgery, herpetic keratitis, or diabetic keratopathy, neutrotrophic keratopathy develops, and persistent corneal defects or trophic ulcer often occur (Dawson & Togni, 1976; Mackie, 1978; Groos, 1997). Therefore, intact corneal innervation is required to maintain the integrity of the normal corneal epithelium, and factors released from sensory nerves appear to play an important role in the physiology of the corneal epithelium.

Using an organ culture of the cornea, we recently found that substance P and insulin-like growth factor-1 (IGF-1) synergistically stimulate corneal epithelial migration (Nishida *et al.*, 1996). Epithelial migration was not affected by the addition of either substance P or IGF-1 alone, but it was stimulated significantly by the combination of substance P and IGF-1. This action of substance P occurred specifically among various kinds of neurotransmitters and tachykinins. Furthermore, substance P and IGF-1 synergistically facilitated corneal epithelial wound closure *in vivo* (Nakamura *et al.*, 1997b). In

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clinical situations, topical application of substance P and IGF-1 was effective in the treatment of severe neurotrophic and anhydrotic keratopathy (Brown *et al.*, 1997).

Substance P is a constituent of sensory nerve fibres and has been postulated to mediate various physiological functions, such as vasodilatation and inflammation (Pernow, 1983; Payan, 1989). Substance P is, however, easily degraded and inactivated by neuropeptidases, such as carboxypeptidases and endopeptidases (Guyon *et al.*, 1979; Matsas *et al.*, 1983; 1984; LeBien & McCormack, 1989). Therefore, finding a smaller and more stable peptide is essential to developing further clinical use of substance P.

In the present study, we wished to identify which portion of the amino acid sequence of substance P might be responsible for this synergism of substance P and IGF-1 in corneal epithelial wound healing. First, based on a previous report showing that a sequence of 5 amino-acids of the C-terminal of substance P is sufficient to produce the synergistic effect with IGF-1 (Nishida *et al.*, 1996), we used an organ culture system of rabbit cornea to examine whether tachykinin-related C-terminal peptides of various lengths are synergistic with IGF-1. Furthermore, we investigated the combined effects of a minimum essential peptide of substance P and IGF-1 on corneal epithelial wound closure of rabbit *in vivo*.

Methods

Materials

Albino rabbits weighing 2–3 kg were obtained from Kitayama Labs, Kyoto, Japan 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. Dispase was from Godo Shusei, Tokyo, Japan. Bovine serum albumin fraction V (BSA) was from Nacalei Tesque, Kyoto, Japan. The [methyl-^{3}H]-thymidine (37 MBq ml^{-1}) was from Amersham Japan, Tokyo, Japan. Plastic multi-well culture dishes were from Costar, Cambridge, MA, U.S.A. Substance P was from Sigma Chemical Co., St. Louis, MO, U.S.A. Human plasma fibronectin and human recombinant IGF-1 were from Collaborative Research, Bedford, MA, U.S.A. Phe-Gly-Leu-Met-NH$_2$ (FGLM), Val-Gly-Leu-Met-NH$_2$ (VGLM), Tyr-Gly-Leu-Met-NH$_2$ (YGLM), Ile-Gly-Leu-Met-NH$_2$ (IGLM), and Gly-Leu-Met-NH$_2$ (GLM) were synthesized from Peptide Institute, Osaka, Japan. Substance P (1-7), Arg-Pro-Lys-Pro-Gln-Gln-Phe, was from Neosystem Laboratories, Strasbourg, France. Care and treatment of animals adhered to the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 86-23).

Epithelial migration

The length of the path of the epithelial migration over the cut stromal surface of a block of cultured rabbit cornea was measured as reported previously (Nishida *et al.*, 1983; Nakamura *et al.*, 1997a). 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 multiwell tissue culture dish (24 wells) with one of the culture media indicated below and incubated at 37°C under humidified 5% CO$_2$ in air. The culture media used were unsupplemented TC-199 (control) or TC-199 containing various agents to be examined. At the end of cultivation, specimens were fixed with a mixture

of glacial acetic acid and absolute ethanol (5 : 95 in volume) 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 haemotoxylin-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 were expressed as the mean ($\pm$s.e.mean) for six determinations.

Preparation of corneal epithelial cells

Corneal epithelial cells were prepared and cultured as described previously (Nishida *et al.*, 1988). In brief, rabbit corneas were excised. The endothelial layers were removed mechanically, and the remaining corneas were incubated with dispase (2 mg ml^{-1} in TC-199) for 1 h. The epithelial layer was harvested as a sheet and washed with TC-199 to remove the debrise. The epithelial layer was incubated with trypsin (0.125%) and EDTA (0.01%) to make single-cell suspensions. The cells were then washed with TC-199 containing 15% FBS.

Characterization of corneal epithelial cells

To confirm the homogeneity and specificity of epithelial cells, we immunostained the cells with anti-cytokeratin antibody (AE1 + AE3, epithelial cell marker) and anti-vementin antibody (V9, fibroblastic cell marker). All of the isolated rabbit corneal epithelial cells were immunoreactive with anti-cytokeratin but not with anti-vementin antibody. As a control, we isolated rabbit corneal fibroblasts, and the cells were immunoreactive with anti-vementin but not with antibody anti-cytokeratin antibody (Figure 1). Therefore, the cells used in our experiments were homogeneous epithelial cells. No contamination of stromal cells, corneal fibroblasts, was observed.

[^{3}H]-thymidine uptake

Corneal epithelial cells were prepared as described above and were cultured on a 96-multiwell culture plate (5 × 10^4 cells well^{-1}) for 4 days in TC-199 containing 15% FBS. The culture medium was then changed to TC-199 containing 1% FBS (low-serum medium) with or without substance P (2 × 10^{-5} M) or Phe-Gly-Leu-Met-NH$_2$ (2 × 10^{-5} M) and/or IGF-1 (10 ng ml^{-1}). No agent was added to the control cultures. As a positive control, 10 ng/ml of EGF was added to the culture medium. Twenty-four hours after the change of the medium, [^{3}H]-thymidine (3.7 KBq well^{-1}, 37 KBq ml^{-1}) was added. The cells were incubated for a further 24 h and harvested, and the radioactivity was measured with a liquid scintillation counter. Data were expressed as means ($\pm$s.e.mean) c.p.m. well^{-1} from four determinations.

Cell attachment assay

The dissociated corneal epithelial cells were cultured in TC-199 culture medium containing 15% FBS for 4 days. The medium was changed to TC-199 containing 1% FBS with or without substance P (2 × 10^{-5} M) or Phe-Gly-Leu-Met-NH$_2$ (2 × 10^{-5} M) and/or IGF-1 (10 ng ml^{-1}), and incubation was continued for another 24 h. Cells were harvested by a 0.125% trypsin and 0.01% EDTA solution. The cells were washed with unsupplemented TC-199, and the cell suspension was adjusted to 1 × 10^4 cells ml^{-1}. This cell suspension (0.1 ml) was plated on each well of a 96-well tissue culture plate, precoated with
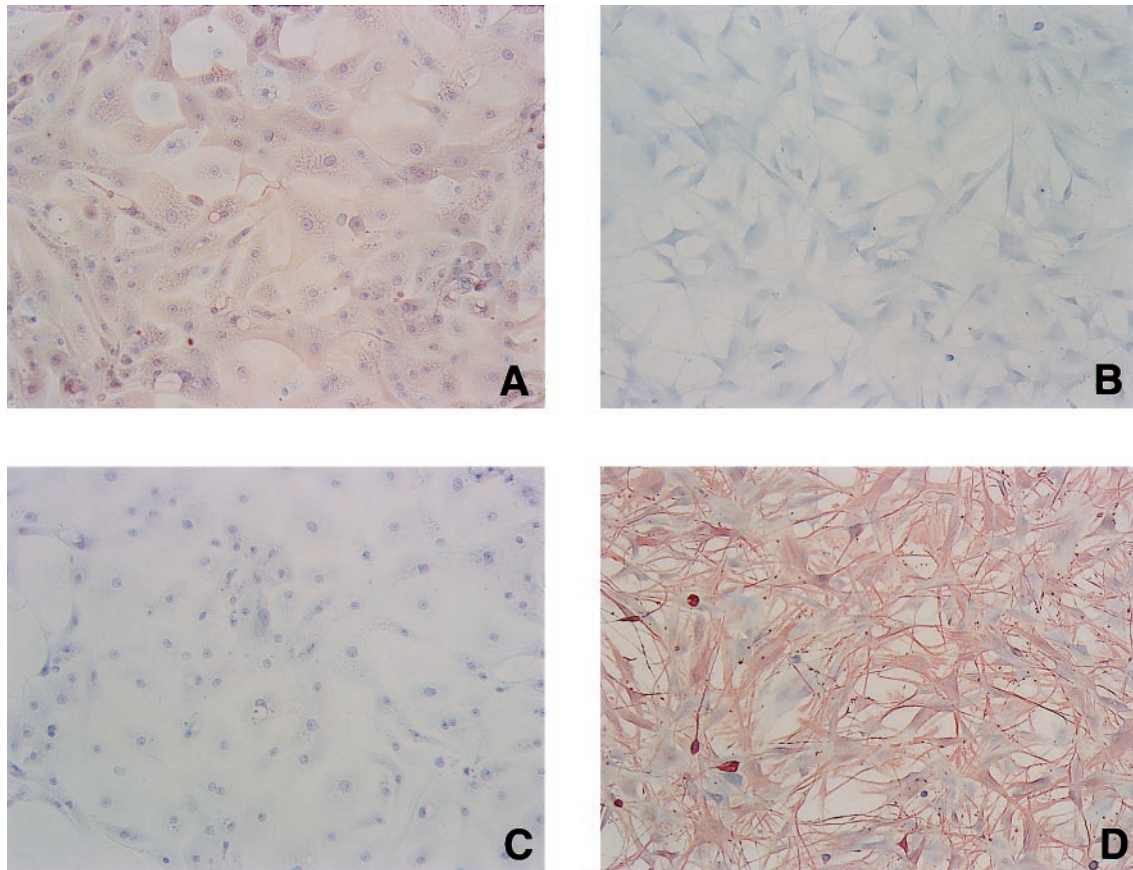
Figure 1 Characterization of rabbit corneal epithelial cells and fibroblasts. Cultured rabbit corneal epithelial cells (A and C) and fibroblasts (B and D) were immunostained with anti-cytokeratin antibody (AE1 + AE3, epithelial cell marker) (A and B) or anti-vementin antibody (V9, fibroblastic cell marker) (C and D).

human plasma fibronectin (10 μg ml^{-1}) plus BSA (1 mg ml^{-1}), or with BSA alone (1 mg ml^{-1}). After 45 min of incubation at 37°C, the cells were fixed with 3.7% formaldehyde and stained with 1% crystal violet. The numbers of attached cells were counted under an inverted phase contrast microscope. Data were expressed as the mean ($\pm$s.e.mean) of the number of attached cells per well in an assay performed in triplicate.

Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was extracted from cultured rabbit corneal epithelial cells with ISOGEN from Nippongene, Toyama, Japan, and quantitated spectrophotometrically by absorption at 260 and 280 nm. The specific mRNA levels were estimated by means of a reverse transcription-polymerase chain reaction protocol using a Takara RNA PCR kit (AMV) ver. 2.1 (Takara Shuzo, Otsu, Shiga, Japan). One half μg of total RNA was reverse transcribed into cDNA by incubation with 0.25 U of avian myeloblastosis virus reverse transcriptase XL in a reaction buffer containing 1/10 volume of 10 × RNA PCR buffer, MgCl$_2$ 5 mM, dNTP mixture 1 mM, randam 9 mers 2.5 pmol ml^{-1} and 1 U of RNase inhibitor in a final volume of 20 μl. The reaction mixture was incubated at 30°C for 10 min, and at 42°C for 30 min, and the reaction was stopped by heating at 99°C for 5 min and then cooling down to 4°C.

A polymerase chain reaction was carried out in a GeneAmp™ PCR System 9600 (Perkin-Elmer, Norwalk, CT, U.S.A.) by adding 80 μl of reaction mixture containing 8 μl of 10 × RNA PCR buffer, 0.025 U of Taq DNA polymerase, and 100 pmol of sense and antisense primers to 20 μl of cDNA solution. The following sequence of primers was used in the PCR reactions: integrin α5 sense: 5′-GGCAGC-TATGGCGTCCCACTGTGG-3′, integrin α5 antisense: 5′-GGCATCAGAGGTGGCTGGAGGCTT-3′, integrin β1 sense: 5′-GTGGTTGCTGGAATTGTTCTTATT-3′, integrin β1 antisense: 5′-TTTTCCCTCATACTTCGGATTGAC-3′, glyceraldehyde 3-phosphate dehydrogenase (G3PDH, internal control) sense: 5′-CGCGGGCCATTCATTGACCTCCAC-TAC-3′, G3PDH antisense: 5′-CGCGGGCTCCTGGAA-GATGGTGAT-3′. These primers led to expected cDNA sequences of 171 base pairs (bp) for integrin α5, 189 bp for integrin β1, and 147 bp for G3PDH (Fini *et al.*, 1995; Ohashi *et al.*, 1995). The condition for the PCR reaction was: 25 cycles, 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The products of amplification were electrophoresed in 1.5% of agarose gel (Sigma Chemical Co., St. Louis, MO, U.S.A.), containing 1 mg per ml of ethidium bromide (Nippongene, Toyama, Japan) and visualized on the u.v. transilluminator. The band area of integrin α5 or β1 and G3PDH was measured, and the band area of integrin α5 or β1 was normalized with the band area of G3PDH, and then the specific integrin α5 or β1 mRNA level was estimated.

In vivo corneal epithelial wound closure

Twenty-four albino rabbits were used. The animals were anaesthetized with an intramuscular injection of ketamine and xylazine and with oxybuprocaine eye drops. A corneal

epithelial wound was produced in each eye according to the method of Cintron and others (Cintron *et al.*, 1979). In brief, filter paper (6 mm in diameter) soaked with n-heptyl alcohol was placed on each cornea for 1 min, and then the damaged epithelial cells were washed out with saline. This method makes a uniform wound, and the denuded area appears quite clean and smooth, with a sharply demarcated wound edge. The basement membrane underlying the epithelium is not damaged (Cintron *et al.*, 1979). This wound-healing model was used to evaluate the growth factors and peptides (Schultz *et al.*, 1988; Takagi *et al.*, 1994; Sotozono *et al.*, 1995).

The animals were divided into six groups (eight eyes per group). The control group was treated with eye drops containing PBS alone; the experimental groups received PBS containing substance P or Phe-Gly-Leu-Met-NH$_2$ at a concentration of 1 mM, or IGF-1 at a concentration of 1 μg ml^{-1}, or a combination of substance P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1. In general, concentrations 10–100 fold higher than the effective doses in *in vitro* experiments are needed to evaluate the effects of growth factors and peptides *in vivo* (Schultz *et al.*, 1988; Takagi *et al.*, 1994; Sotozono *et al.*, 1995). Based on our *in vitro* experiments, the concentrations of substance P, Phe-Gly-Leu-Met-NH$_2$ and IGF-1 used in this study were 50 fold and 100 fold higher, respectively, than their *in vitro* effective doses. All eyes received one drop of the solution immediately after the n-heptyl alcohol treatment and again at 2, 4, 6, 8, 10, 24, 26, 28, 30, 32, and 34 h after debridement. The epithelial defects were stained with one drop of 2% fluorescein and photographed immediately following wounding and at 6, 12, 18, 24, 30, 36, and 48 h after wounding. The area of each epithelial defect was measured on the photographs with a computer-assisted digitizer and expressed as the mean, in square millimeters, for the eyes in each group. The healing rate of each eye was calculated with linear regression analysis of the data collected at 12, 18, 24, and 30 h and expressed in square millimeters per hour. The experiment was carried out in a double-masked fashion to avoid any bias. The rate of healing for each group was expressed as the mean ($\pm$ s.e.mean). Pupil size was measured by Haab's pupillometer before instillation of eye drops at 10, 24, 34, and 48 h after debridement.

Statistical analysis

Statistical analysis was carried out by an unpaired Student's *t*-test for comparison of two groups, and by the Dunnett's multiple comparison test for comparison of three or more groups; pupil size was compared using a Scheffe-type multiple comparison test.

Results

Based on a previous report showing that the sequence of 5 amino acids of the C-terminal of substance P is sufficient to produce the synergistic effect with IGF-1 (Nishida *et al.*, 1996), we examined whether tachykinin-related C-terminal peptides of various lengths are synergistic with IGF-1 (Figure 2). The addition of substance P (SP, 11 amino-acid peptides, SP1-11), Phe-Gly-Leu-Met-NH$_2$ (FGLM, 4 amino-acid sequence at C-terminal of substance P, SP8-11), Val-Gly-Leu-Met-NH$_2$ (VGLM, 4 amino-acid sequence at C-terminal of neurokinin A, neurokinin B and kassinin), Tyr-Gly-Leu-Met-NH$_2$ (YGLM, 4 amino-acid sequence at C-terminal of physalaemin), Ile-Gly-Leu-Met-NH$_2$ (IGLM, C-terminal of eledoisin), or Gly-Leu-Met-NH$_2$ (GLM, 3 amino-acid sequence at

common C-terminal of tachykinins) at a concentration of 2×10^{-5} M did not affect corneal epithelial migration. However, in the presence of IGF-1 at a concentration of 10 ng ml^{-1}, substance P and Phe-Gly-Leu-Met-NH$_2$ are synergistic in stimulating corneal epithelial migration. No synergistic effects with other peptides (Val-Gly-Leu-Met-NH$_2$, Tyr-Gly-Leu-Met-NH$_2$, Ile-Gly-Leu-Met-NH$_2$, nor Gly-Leu-Met-NH$_2$) were observed. In addition, the 7 amino-acid peptides at the N-terminal of substance P, SP1-7, which remained peptides of the Phe-Gly-Leu-Met-NH$_2$, also did not affect corneal epithelial migration, whether or not IGF-1 was present (Figure 3). These results demonstrated that the sequence of the 4 amino-acids of the C-terminal of substance P is the minimum number of peptides required to produce the synergistic effect with IGF-1. Furthermore, among tachykinins (neurokinin A, neurokinin B, kassinin, physalaemin, eledoisin), only the C-terminal of substance P demonstrated a synergistic effect with IGF-1 on corneal epithelial migration when evaluated in our *in vitro* assay.

We then examined whether the synergistic effect of Phe-Gly-Leu-Met-NH$_2$ with IGF-1 was concentration dependent (Figure 4). When various concentrations of Phe-Gly-Leu-
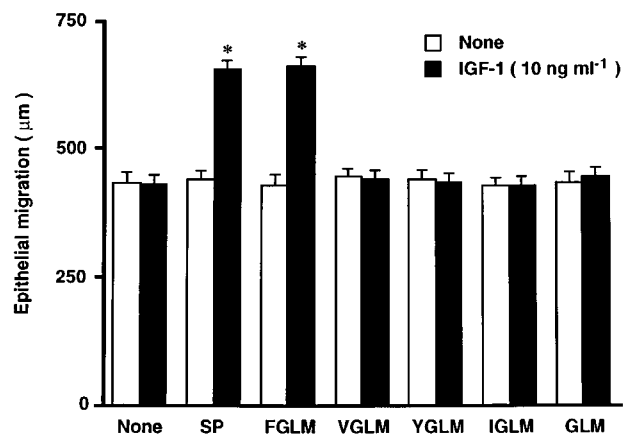
Figure 2 Synergistic effects with various types of C-terminal of tachykinin peptides with IGF-1 on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing various kinds of peptides (2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}). Error bars represent the s.e.mean from six determinations. *$P < 0.01$ versus the corneal blocks cultured without IGF-1.
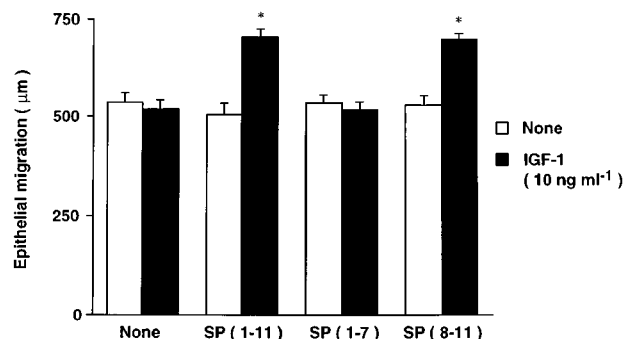
Figure 3 Synergistic effects with sequence of 7 amino-acids of N-terminal of substance P (SP), SP(1–7), and sequence of 4 amino-acids of C-terminal of SP, SP(8–11), with IGF-1 on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing peptides (2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}). Error bars represent the s.e.mean from six determinations. *$P < 0.01$ versus the corneal blocks cultured without IGF-1.

Met-NH$_2$ ($0 - 2 \times 10^{-5}$ M) were added in the absence of IGF-1, no change in the length of epithelial migration was observed. In the presence of IGF-1 at a concentration of 10 ng ml^{-1}, however, the length of the path of epithelial migration increased in proportion to the concentration of Phe-Gly-Leu-Met-NH$_2$. At a concentration of 1 or 2×10^{-5} M of Phe-Gly-Leu-Met-NH$_2$, the length of epithelial migration was significantly higher in the corneal blocks cultured with IGF-1 than in those cultured without IGF-1 ($P < 0.01$) (Figure 4A). Corneal blocks were then cultured in varying concentrations of IGF-1 ($0 - 10$ ng ml^{-1}) in the absence or presence of Phe-Gly-Leu-Met-NH$_2$ (2×10^{-5} M) for 24 h (Figure 4B). The addition of IGF-1 did not affect the length of epithelial migration in the absence of Phe-Gly-Leu-Met-NH$_2$. However, in the presence of Phe-Gly-Leu-Met-NH$_2$, the length of epithelial migration increased in proportion to the concentration of IGF-1. At concentrations of 1 or 10 ng ml^{-1} of IGF-1 with Phe-Gly-Leu-Met-NH$_2$, the length of epithelial migration was significantly higher than the length of epithelial migration in the corneal blocks cultured without Phe-Gly-Leu-Met-NH$_2$ alone ($P < 0.01$). These results demonstrate that the synergistic

effects of Phe-Gly-Leu-Met-NH$_2$ with IGF-1 are mutually concentration dependent.

Corneal epithelial migration depends on the increase in the proliferation of the cells and/or an increase in the attachment to extracellular matrix proteins, such as fibronectin. Therefore, we examined whether Phe-Gly-Leu-Met-NH$_2$ and IGF-1 acted in a synergistic way on [^{3}H]-thymidine uptake and/or the attachment of the epithelial cells to the fibronectin matrix.

The addition of only IGF-1 (10 ng ml^{-1}) to cultured corneal epithelial cells did not affect [^{3}H]-thymidine uptake. When substance P or Phe-Gly-Leu-Met-NH$_2$ (2×10^{-5} M) was added to corneal epithelial cells, no change in [^{3}H]-thymidine uptake was observed, whether IGF-1 was absent or present (Table 1). When EGF (10 ng ml^{-1}) was added simultaneously, [^{3}H]-thymidine uptake was $13{,}433 \pm 549$ c.p.m./well, which demonstrated that the cells were not under contact inhibition. Thus, no synergistic effects of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 on corneal epithelial proliferation were observed.

We then examined the synergistic effects of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 on the attachment of corneal epithelial cells to the fibronectin matrix (Figure 5). Whether the cells were cultured in the absence or presence of substance P or Phe-Gly-Leu-Met-NH$_2$ and/or IGF-1, the number of cells attached to the control plates coated with BSA remained low, and there was no significant difference in the number of cells attached (Figure 5B). When the cells were cultured with or without substance P, Phe-Gly-Leu-Met-NH$_2$, or IGF-1 alone, no significant effect of fibronectin-coated plates on cellular attachment was observed. However, when the cells were cultured in the presence of substance P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1, the number of cells attached on fibronectin-coated plates increased significantly (Figure 5A). These results demonstrate that substance P, Phe-Gly-Leu-Met-NH$_2$, or IGF-1 alone did not affect the attachment of corneal epithelial cells to a fibronectin matrix, but they act synergistically to promote attachment of the cells to a fibronectin matrix.

Using a RT–PCR, we then investigated whether the combination of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 up-regulate the expression of integrin $\alpha 5 \beta 1$, a fibronectin receptor on corneal epithelial cells (Figure 6). When the cells were treated with or without substance P, Phe-Gly-Leu-Met-NH$_2$, or IGF-1 alone for 2 h, no significant change was observed in the levels of transcripts of either $\alpha 5$ or $\beta 1$ integrin. When the cells were treated in the presence of both substance P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1, however, the levels of both $\alpha 5$ and $\beta 1$ integrin mRNA increased significantly, resulting in about 3 and 1.5 fold increases, respectively (Figure 7A and B). The level of G3PDH mRNA did not affect any treatments, indicating that the expression of $\alpha 5$ and $\beta 1$ integrin were
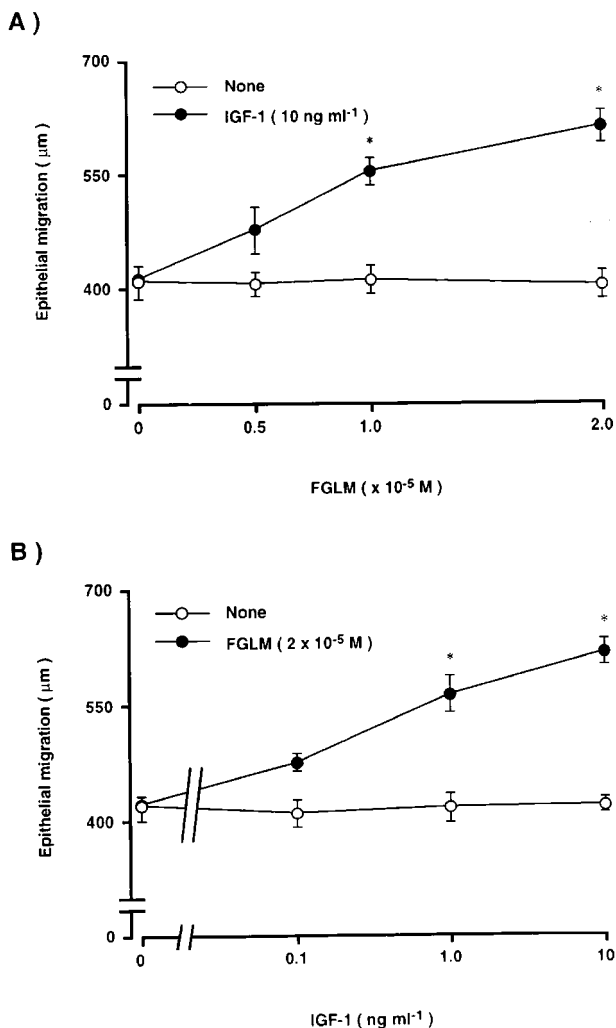

Figure 4 Synergistic effects of Phe-Gly-Leu-Met-NH$_2$ (FGLM) with IGF-1 on corneal epithelial migration. Corneal blocks were cultured for 24 h in TC-199 containing: (A) FGLM (0.5, 1, 2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}); (B) IGF-1 (0.1, 1, 10 ng ml^{-1}) in the absence or presence of FGLM (2×10^{-5} M). Error bars represent the s.e.mean from six determinations. *$P < 0.01$ versus the corneal blocks cultured without FGLM or IGF-1.

Table 1 Synergistic effects of substance p (SP) or Phe-Gly-Leu-Met-NH$_2$ (FGLM) with IGF-1 on corneal epithelial cell proliferation

Addition	[^{3}H]-thymidine uptake (c.p.m. well^{-1})	
	None	*IGF-1 (10 ng ml^{-1})*
None	313 ± 26	304 ± 57
SP (2×10^{-5} M)	307 ± 69	305 ± 38
FGLM (2×10^{-5} M)	311 ± 44	319 ± 54

Corneal epithelial cells were cultured in TC-199 containing SP or FGLM (2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}). When EDF (10 ng ml^{-1}) was added simultaneously, [^{3}H]-thymidine uptake was 13434 ± 549 c.p.m. well^{-1}. Data represent means $\pm$ s.e.mean from four determinations.
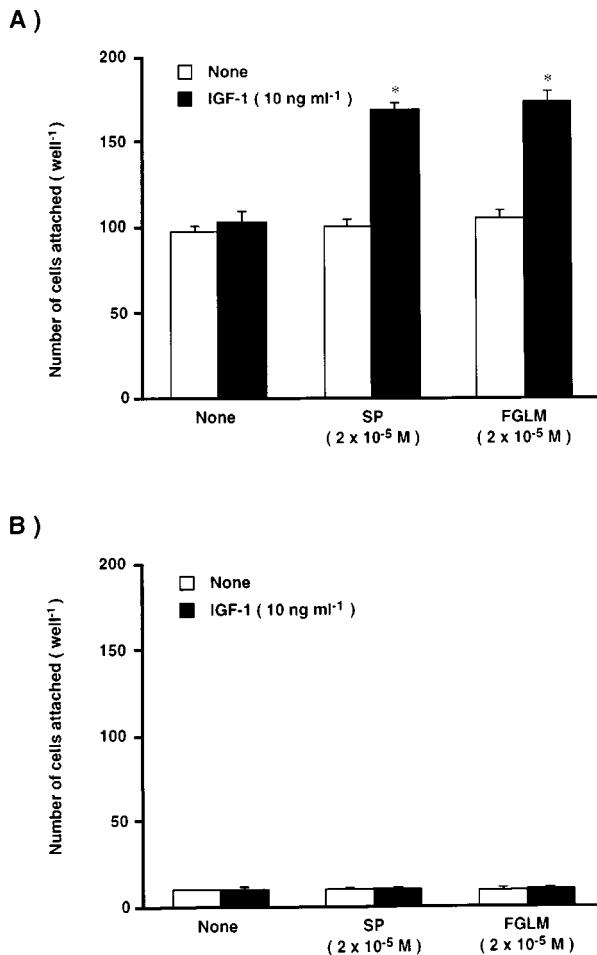
A)

B)

Figure 5 Synergistic effects of substance P (SP) or Phe-Gly-Leu-Met-NH$_2$ (FGLM) with IGF-1 on corneal epithelial cell attachment to the fibronectin matrix. Corneal epithelial cells were cultured for 24 h in TC-199 containing SP or FGLM (2×10^{-5} M) in the absence or presence of IGF-1 (10 ng ml^{-1}). The dissociated epithelial cells were then plated on the fibronectin (coated at 10 μg ml^{-1}) plus BSA (1 mg ml-1) (A) or BSA matrix (B), and incubated for 45 min. Error bars represent the s.e.mean from three determinations. *$P < 0.01$ versus the corneal epithelial cells cultured without IGF-1.
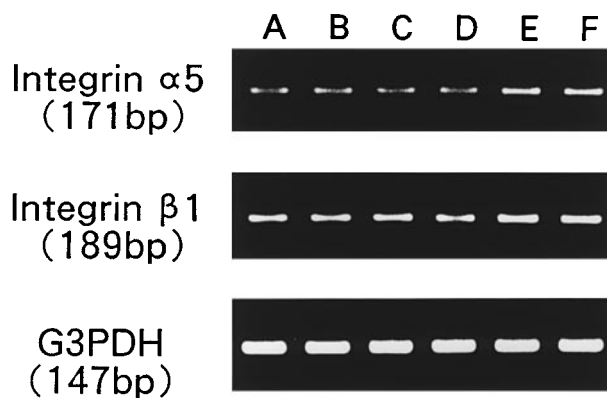
specifically up-regulated by the combination of substnace P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1.

We next, investigated the combined effects of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 on corneal epithelial wound closure *in vivo*. No significant difference in the size of the wound area was observed among the control (PBS) and experimental groups immediately after debridement (Figure 8). During the first 6 h after debridement, healing was rather slow, and no differences were observed between the control group and the groups treated with substance P or Phe-Gly-Leu-Met-NH$_2$ and/or IGF-1. Twelve to 30 h after debridement, the rate of wound closure accelerated and was almost linear. The administration of substance P, Phe-Gly-Leu-Met-NH$_2$, or IGF-1 alone did not change the rate of closure compared with that of the control group. However, the combination of substance P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1 significantly increased this rate compared with that of the control group. After 36 h, the healing rate slowed in both the control and experimental groups.

The mean healing rates ($\pm$ s.e.mean, in square millimeters per hour) were 0.87 ± 0.03 (PBS eye drops alone), 0.92 ± 0.06 (substance P alone), 0.90 ± 0.06 (Phe-Gly-Leu-Met-NH$_2$
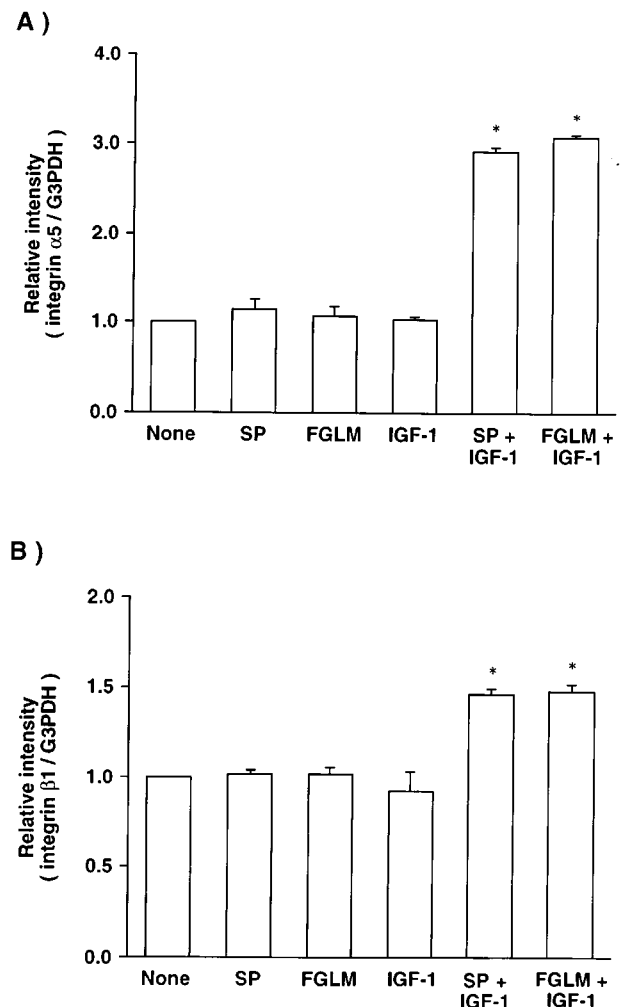
A)

B)

Figure 7 Effects of substance P (SP) or Phe-Gly-Leu-Met-NH$_2$ (FGLM) and/or IGF-1 on the gene expression of integrin α5 (A) and integrin β1 (B) in cultured cornea epithelial cells using an RT–PCR. Corneal epithelial cells were cultured for 2 h in TC-199 containing SP or FGLM (2×10^{-5} M) and/or IGF-1 (10 ng ml^{-1}), and then RT–PCR was performed. Error bars represent the s.e.mean from three determinations. *$P < 0.05$ versus the corneal epithelial cells cultured without SP, FGLM, or IGF-1.
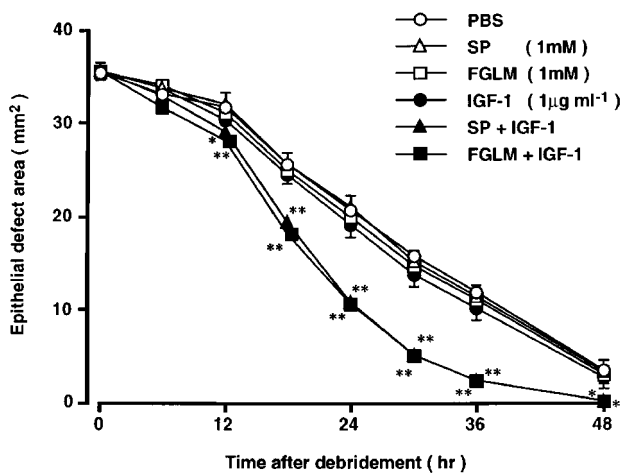

Figure 6 RT–PCR transcription analysis of integrin α5 and β1 expression in corneal epithelial cells after treatment with substance P (SP) or Phe-Gly-Leu-Met-NH$_2$ (FGLM) and/or IGF-1. Ethidium bromide-staining gels of PCR amplified products of reverse-transcribed epithelial cell RNA. A: None, B: SP (2×10^{-5} M), C: FGLM (2×10^{-5} M), D: IGF-1 (10 ng ml^{-1}), E: SP plus IGF-1, F: FGLM plus IGF-1.

Figure 8 Effects of substances P (SP), Phe-Gly-Leu-Met-NH$_2$ (FGLM), and/or IGF-1 on the area of the cornea epithelial defect. There were eight eyes per treatment group. Data are expressed as means $\pm$ s.e.mean. $*P < 0.05$; $**P < 0.01$ compared with control group.

Table 2 Effects of subtance P (SP), Phe-Gly-Leu-Met-NH$_2$ (FGLM) and/or IGF-1 on pupil size[†]

| | *Pupil size* (mm) | | | |
	10 h	24 h	34 h	48 h
PBS	4.81	4.94	5.19	5.56
SP (1 mM)	1.88**	5.19	2.81*	6.19
FGLM (1 mM)	4.81	5.06	5.38	5.50
IGF-1 (1 μg/ml)	4.63	4.94	5.06	5.44
SP + IGF-1	1.88**	5.13	2.75*	6.13
FGLM + IGF-1	4.75	5.06	5.25	5.56

†Pupil size was measured by Haab's pupillometer before instillation of eye drops at 10, 24, 34, and 48 h after debridement. There were eight eyes per treatment group. Data are expressed as means. Statistical analysis by Scheffe-type multiple comparison test: $*P < 0.05$, $**P < 0.01$ against PBS-treated controls.

alone), 0.92 ± 0.06 (IGF-1 alone), 1.34 ± 0.04 (substance P and IGF-1), and 1.28 ± 0.03 (Phe-Gly-Leu-Met-NH$_2$ and IGF-1). No significant difference was observed between the control group receiving only PBS and the group receiving substance P, Phe-Gly-Leu-Met-NH$_2$, or IGF-1. The mean healing rate in the group treated with substance P or Phe-Gly-Leu-Met-NH$_2$ and IGF-1 was significantly higher than in control eyes ($P < 0.01$).

In contrast, instillation of substance P significantly decreased pupil size, whether or not IGF-1 was present. However, Phe-Gly-Leu-Met-NH$_2$ and/or IGF-1 did not affect pupil size (Table 2).

Discussion

The pathophysiological role of innervation in the cornea is not yet fully understood. However, loss of corneal sensation often leads to the destruction of normal integrity of the cornea. Persistent corneal epithelial defects or delayed epithelial wound healing often occur in patients whose corneal sensation is diminished as a result of diseases such as herpes simplex, herpes zoster, and diabetes mellitus. Furthermore, corneal

ulceration may often develop in the anaesthetized eye (Dawson & Togni, 1976; Mackie, 1978; Groos, 1997). On the basis of these clinical observations, we believe that corneal innervation may be important for maintaining normal integrity of the cornea. Nonetheless, the definitive role of the neural function in corneal pathophysiology has not yet been clarified.

We recently demonstrated that substance P and IGF-1 acting alone do not influence corneal epithelial wound healing but that they act synergistically to stimulate epithelial wound healing *in vitro* and *in vivo* (Nishida *et al.*, 1996; Nakamura *et al.*, 1997b). Furthermore, this synergistic effect of substance P and IGF-1 was found to be mediated through the NK-1 receptor for substance P on corneal epithelial cells (Nakamura *et al.*, 1997c). We tried to identify the minimum essential amino-acid sequence of substance P to develop the clinical use of substance P for the treatment of neurotrophic keratopathy, because substance P is easily degraded and inactivated by peptidases in the body (Guyon *et al.*, 1979; Matsas *et al.*, 1983; 1984; LeBien & McCormack, 1989). The present results demonstrated that Phe-Gly-Leu-Met-NH$_2$, the sequence of 4 amino-acids from the C-terminal of substance P, is the minimum sequence necessary to generate a synergistic effect with IGF-1 on corneal epithelial migration *in vitro* and epithelial wound closure *in vivo*.

Tachykinins have a common C-terminal sequence of 5 amino-acids: Phe-X-Gly-Leu-Met-NH$_2$ (FXGLM). Among the various amino-acids at position 8 (marked by an X in the sequence above) in the tachykinins examined, only Phe-Gly-Leu-Met-NH$_2$ exhibited a synergistic effect with IGF-1 on corneal epithelial migration. Furthermore, unlike substance P or Phe-Gly-Leu-Met-NH$_2$, Gly-Leu-Met-NH$_2$, the tachykinin C-terminal sequence of 3 amino-acids, did not demonstrate any synergistic effect with IGF-1. On the other hand, most of the biological effects of substance P have been thought to be mediated by the C-terminal of substance P, but the N-terminal of substance P, such as substance P $(1-7)$, also has been shown to produce biological effects and often has produced effects opposite from those produced by the C-terminal of substance P (Hall & Stewart, 1984; Igwe *et al.*, 1990; Larson & Sun, 1992; Yukhanaov & Larson, 1994). Substance P $(1-7)$ did not, however, demonstrate any synergistic effect with IGF-1. Therefore, this C-terminal sequence of 4 amino-acids would appear to be essential for expressing the synergistic effect with IGF-1. Although we cannot explain why the C-terminal sequence of 4 amino-acids of tachykinins other than substance P does not produce a synergistic effect with IGF-1, it could be that the amino acid at position 8 is critical for producing the synergistic effect with IGF-1 on corneal epithelial wound healing.

An intact epithelium is very important to the survival of a multicellular organism. The epithelium serves as the first defense against the external environment, maintaining the internal homeostasis. Continuous renewal through an active repair system is one of the most important mechanisms for the maintenance of epithelial integrity. There are three phases involved in the process of corneal epithelial wound healing: migration or mobilization, proliferation or mitosis, and differentiation of the epithelial cell (Nishida, 1993; Dua *et al.*, 1994; Gipson & Inatomo, 1995; Woodley, 1996). Among these events in epithelial wound healing, epithelial cell migration is the initial step in successful and complete resurfacing of a defect. During this step, attachment of the corneal epithelial cells to the underlying provisional fibronectin matrix is required for spreading and migration. Fibronectin and fibronectin receptor (integrin $\alpha 5 \beta 1$) play significant roles in this process. As we reported in this study, both Phe-Gly-Leu-

Met-NH$_2$ and substance P act synergistically with IGF-1 on cell migration, attachment to a fibronectin matrix, and expression of integrin $\alpha5\beta1$. Furthermore, in an *in vivo* study, we observed the synergistic effect of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 during the initial phase of healing 12 h after debridement. However, no synergistic effect of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 on epithelial cell proliferation was observed. Therefore, these results demonstrate that the synergistic effect of Phe-Gly-Leu-Met-NH$_2$ and IGF-1 on corneal epithelial wound healing results from an effect on corneal epithelial migration rather than on proliferation. This synergism would appear to represent an important cooperative action of neural and humoral regulation on corneal epithelial wound healing.

Substance P 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), but neurotransmitters were inactivated by enzymatic degradation and re-uptake. In the case of substance P, enzymatic degradation is the main mechanism of inactivation. Substance P is degraded by cell-surface and soluble peptidases, especially cell-surface peptidases, such as aminopeptidases, carboxypeptidases and endopeptidases, which have been investigated thoroughly (Krause, 1985; Kenny & Hooper, 1991). Indeed, ocular tissues and fluids showed various types of peptidase activity (Pahlitzsch & Sinha, 1985; Stratford & Lee, 1985; Kashi & Lee, 1986; Sharma & Ortwerth, 1987; Igic, 1993; Coupland *et al.*, 1994), and substance P was degraded and inactivated by these peptidases. Among these peptidases, neural endopeptidase (NEP) has been purified and well characterized about attack sites of substance P (Matsas *et al.*, 1983; 1984; Skidgel *et al.*, 1984; Roques *et al.*, 1993). In general, NEP is hydrolyzed at bonds Gln-Phe (positions 6 and 7), Phe-Phe (positions 7 and 8), and Gly-Leu (positions 9 and 10) of substance P. However, Lee and others (Lee *et al.*, 1981) reported that no attack was observed at the bond at positions 9 and 10, and substrate specificity of NEP might have species or regional variations. As we reported in this paper, 4 amino-acid sequences at the C-terminal or substance P might not be degraded by enzymatic cleave. Thus, Phe-Gly-Leu-Met-NH$_2$ might be stable, when it is given to the eye in place of a whole molecule of substance P.

Our present study also shows that topical application of substance P induces mitosis, one of the pharmacological actions of substance P, but application of Phe-Gly-Leu-Met-NH$_2$ did not. Therefore, the use of Phe-Gly-Leu-Met-NH$_2$ may be excluded from some adverse effects of substance P because mitosis induces darkness. Further studies are needed to understand the stability and pharmacological actions of Phe-Gly-Leu-Met-NH$_2$ in the corneal epithelium.

Substance P has been reported to stimulate the migration and proliferation of skin fibroblasts through NK-1 receptors (Ziche *et al.*, 1990; Parenti *et al.*, 1996). Although we did not examine the effect of substance P on corneal stromal cells, the same stromal cells are able to produce growth factors, which in turn could stimulate epithelial cells. In the present studies, however, substance P and Phe-Gly-Leu-Met-NH$_2$ acting alone did not affect the migration and proliferation of epithelial cells. As shown in Figure 1, the cells used in our experiments were corneal epithelial cells, not corneal fibroblasts. Therefore, the mechanisms that trigger the action of substance P might be different in corneal epithelial cells and fibroblasts.

The corneas contain sensory nerve fibres containing substance P immunoreactivity (Miller *et al.*, 1981; Tervo *et al.*, 1981; 1982; Stone & Kuwayama, 1985). Previous studies have demonstrated a correlation between reduced substance P levels in the cornea and denervation of the trigeminal nerve to the eye (Butler *et al.*, 1980; Unger *et al.*, 1981; Keen *et al.*, 1982). However, like previous studies, the present study has demonstrated that adding only substance P did not influence corneal epithelial wound healing (Nishida *et al.*, 1996; Nakamura *et al.*, 1997b). Kingsley & Murfurt (1997) also reported that substance P alone had no significant effect on corneal epithelial wound healing. Cooperative action of the neural and humoral factors in the corneal epithelium may be important in improving the corneal disorder resulting from corneal denervation. Our present study strongly suggests the possibility of using Phe-Gly-Leu-Met-NH$_2$ and IGF-1 to treat pathological conditions such as herpetic keratitis or diabetic keratopathy, which are due to delayed wound healing following corneal desensitization.

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