Expression of Neurotensin Receptors in Human Corneal Keratocytes

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PURPOSE. The purpose of the study was to investigate whether cultured human keratocytes express the neurotensin receptors (NTR1, NTR2, and NTR3), to determine the presence of neurotensin (NT) in keratocytes, and to assess the influence of NT on these cells.

METHODS. Human keratocytes were cultured in medium treated with various concentrations (10⁻⁷ to 10⁻⁵ M) of JMV449 (a weakly degradable NT agonist). Cell proliferation and viability were analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay. Apoptosis was studied by nucleus labeling with a fluorescent dye and cold light fluorometry. NT, NTR1, NTR2, and NTR3 mRNA were detected in human keratocytes by means of reverse transcriptase–polymerase chain reaction (RT-PCR). NTR1 protein was detected by Western blot analysis. Functionality of NTR1 was assessed by intracellular calcium (Ca²⁺) measurement with a dynamic imaging microscopy system.

RESULTS. RT-PCR and Western blot analysis showed the expression of the NTR1 (mRNA and protein) and NTR3 mRNA in human corneal keratocytes. NT and NTR2 mRNA were undetectable. JMV449 induced a rapid and transient [Ca²⁺]i increase in human corneal keratocytes that was blocked by the specific antagonist SR48692. JMV449 significantly increased cell proliferation and viability after 72, 96, and 120 hours of culture, with a maximum effect at 10⁻⁷ M (P < 0.005). Finally, JMV449 decreased keratocyte apoptosis, whatever the concentration used (P < 0.005).

CONCLUSIONS. These results indicate that cultured human keratocytes express NTR1 and NTR3 and that NT may exert physiological effects on cornea such as regulation of keratocyte proliferation and apoptosis. (Invest Ophthalmol Vis Sci. 2002; 43:1765–1771)

The cornea is an essential anatomic and physiological barrier between the eye and the external environment. To protect the eye from injury, the corneal epithelium and the keratocytes of the anterior stroma are provided with a rich nerve supply. In addition to their sensory functions, corneal nerves exert important trophic effects on the cornea, including maintenance of epithelial integrity, modulation of cell proliferation and mitosis, stimulation of ion transport, and regulation of wound healing after corneal injuries. The mechanisms by which the nerves mediate their effects remain poorly understood. However, the implication of biologically active neuropeptides has been hypothesized. Recent immunohistochemical investigations have shown an important peptidergic innervation of the cornea. Furthermore, several studies suggest that substance P and calcitonin gene-related peptide (CGRP) exert important trophic functions on the cornea and promote corneal wound healing. However, the role of other neuropeptides has not been strongly investigated.

Neurotensin (NT) is a 13-amino-acid peptide originally isolated from extracts of the bovine hypothalamus. Similar to many other neuropeptides, it has the dual functions of neurotransmitter and neuromodulator in the central nervous system (CNS) and of local hormone in the peripheral tissues. In mammals, NT is widely distributed throughout the CNS and digestive tract (enteroendocrine cells of the small intestine), where it acts as a growth factor on a variety of normal or cancer cells. The actions of NT are mediated by the stimulation of several specific receptors (NTRs). Three specific membrane receptors have been described so far. NTR1 shows a high affinity for NT, whereas NTR2 exhibits a low affinity. NTR1 and NTR2 belong to the family of G protein–coupled receptors (GPCRs). NTR3 is an entirely new type of neuropeptide receptor and is identical with gp95/sortilin, a 100-kDa protein with a single transmembrane domain.

Very little information is available on the expression and role of NT and its receptor in the eye. NT has been detected in the iris and ciliary body of several mammalian species and may have a role in the regulation of intraocular pressure. 12–17 Neurotensin-like immunoreactivity has been reported in the amacrines of the chicken retina. These cells are believed to form a part of the retinal dark–light switch and appear to be involved in light adaptation.

NT or its receptors have never been described in the cornea. However, the presence of NT immunoreactivity in the trigeminal nucleus, 20 known to innervate corneal tissue, prompted us to investigate in vitro (1) whether cultured human keratocytes express NTR1, NTR2, and NTR3; (2) to determine the presence of NT in keratocytes; and (3) to assess the possible effects of this peptide on keratocyte proliferation and apoptosis.

MATERIALS AND METHODS

Human Keratocyte Culture

This study was performed according to the tenets of the Declaration of Helsinki. Human keratocyte primary cultures were obtained using human donor corneas that were discarded before transplantation because of low endothelial cell counts. Stromal explants were obtained as previously described. 21 The culture medium consisted of 1:1 mixture of TC199 and HamF12 media (THF; Gibco Life Technology, Cergy-Pontoise, France) with 2.5% fetal calf serum (FCS; Gibco), 20 μg/mL insulin (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), 20...
µg/mL L-ascorbic acid (Sigma-Aldrich Chimie), 250 ng/mL heparin (Leo, Saint Quentin en Yvelines, France), 10 ng/mL aFGF (Sigma-Aldrich Chimie), 0.4 mg/mL sulfate chondroitin (Sigma-Aldrich Chimie), 2 mM l-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin. First-, second-, and third-passage keratocytes were used in all the experiments. The cells were incubated in THF at 37°C (5% CO₂) and allowed to attach to the bottom of the well for 24 hours before addition of JMV449. The control group consisted of keratocytes cultured in THF with no JMV449. The culture media were renewed every day. Cultured keratocytes were studied daily by means of phase-contrast microscopy.

Drug Preparation and Addition
JMV449 was purchased from Neosystem (Strasbourg, France). JMV449 is a potent and stable pseudopeptide NT agonist. It was dissolved in PBS and serially diluted in the culture medium. On the second day, THF was replaced by THF containing various concentrations of JMV449 (10⁻⁷, 10⁻⁸, or 10⁻⁹ M). JMV449 was thus added every day for 3 to 5 days to the culture media.

SR48092 (Sanofi Synthelabo, Toulouse, France), is a potent non-peptide NTR1 antagonist that has been shown to block a number of central and peripheral effects of NT. SR48692 is also a partial agonist of NTR2. It was dissolved in dimethyl sulfoxide (DMSO) and diluted in THF for intracellular calcium ([Ca²⁺]i) measurement experiments.

RNA Preparation
Total RNA extraction was performed on keratocytes taken in primary cultures and at the first and second subcultures from two separate stromal explants. RNA extraction was performed by the acidic phenol-chloroform guanidine thiocyanate method. Total RNAs were suspended in sterile deionized diethylpyrocatearbanate (DEPC) treated water and aliquots were prepared and stored at −80°C. Total RNA recovery was measured by spectrophotometric absorbance at 260 nm.

Reverse Transcription–Polymerase Chain Reaction for NTR
Total RNA (5 µg) was reverse transcribed in a 30-µL reaction mixture containing 20 mM Tris-Cl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 10 mM dithiothreitol (DTT), 1 mM concentration of each dNTP, 1 µg oligo dN and 1 µg oligo dT, 24 U RNAsin (Promega, Madison, WI) and 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen, San Diego, CA) at 37°C for 1 hour. A specific oligonucleotide, GCCAGTAGAAGAG, was used to transcribe NTR1 mRNA. The PCR amplification was performed on 1:5 (vol/vol) of the RT reaction in a mixture containing 16 mM Tris-Cl (pH 8.3), 40 mM KCl, 1 mM MgCl₂, 0.2 mM concentration of each dNTP, 50 pmol of the sense primer, 50 pmol of the antisense primer, and 1 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a final volume of 50 µL. The PCR primers used were: sense (S)-NTR1, 5'-CGTGAGGCTGTAACCTCTA-3', and antisense (AS)-NTR1, 5'-ACTGGCTATCGGAATGTAGT-3' for NTR1; S-NTR2, 5'-TGACCAACAGCTCCTTAT-3', and AS-NTR2, 5'-GGCCAGGTTCCATATCTTC-3' for NTR2; S-NTR3, 5'-TGTTACAGCAGACGGAACC-3', and AS-NTR3, 5'-CTGGTTGGAATCCATATCAGCCA-3' for NTR3; and S-NT, 5'-CTGGTTGGAATCCATATCAGCCA-3', and AS-NT, 5'-ACCAGAAGGCGGCTACTATC-3' for NT. PCR products for NTR1, NTR2, and NT were 637, 396, 537, and 416 nucleotides, respectively.

The amplification profile was divided into denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute 30 seconds. The 35 cycles were preceded by denaturation at 95°C for 5 minutes and immediately followed by a final extension at 72°C for 10 minutes. The amplification was performed in a DNA thermal cycler (Gene Amp PCR system 9700; Perkin-Elmer Cetus).

PCR Product Analysis
PCR-samples (10 µL) were electrophoresed on 1% agarose gels in 90 mM Tris borate and 2 mM EDTA buffer. We routinely introduced a 100-bp DNA ladder (Invitrogen) size marker. Gels were stained with ethidium bromide and photographed under a UV lamp (665 film; Polaroid, Cambridge, MA).

RT-PCR Control Analyses
We routinely introduced a negative control for all assays to confirm the absence of contamination. For these controls, RNA was omitted from the RT reaction mixture, and the reverse transcription was performed as described. PCR amplification was performed in the same conditions as for the samples. Human MCF7, known to express NTR1 and CHO34 cells, were used as the positive control. Hippocampus mRNA was used as the positive control for NT and NTR2.

Western Blot Analysis
Cultured keratocytes obtained from two corneas were lysed using 0.6 mL lysis buffer (10 mM Tris/Cl, 1% SDS [pH 7.4]). Protein concentration was quantified using the bicinchoninic acid protein assay, according to the manufacturer’s instructions (Pierce, Interchim, France). Ten and 20 µg of each protein extract were loaded onto 10% SDS-PAGE gels. Gels were blotted onto a polyvinylidene difluoride (PVDF) membrane and incubated overnight with goat polyclonal antibody against NTR1 (1:1000; Tebu, Le Perrey en Yvelines, France) or anti-actin antibody (1:1000; Sigma-Aldrich Chimie), followed by rabbit anti-goat coupled to horseshadish peroxidase (1:20,000; Sigma-Aldrich Chimie). The reactivity was evaluated using Western blot detection reagents (ECL+PLUS; Amersham Pharmacia Biotech, Saclay, France), visualized with enhanced chemiluminescence and hyperfilm (Amersham), and quantified using a phosphorescence imager and manufacturer-provided software (PhosphorImager and Image Quant, respectively; Molecular Dynamics, Sunnyvale, CA). Relative levels of proteins were normalized against an actin protein band.

Determination of Intracellular Calcium
The [Ca²⁺]i concentration was measured in response to activation of NTR1 receptors, using a dynamic imaging microscopy system (Quanticell 700; Visitech Int. Ltd., UK) with 15 to 20 keratocytes per field, as described earlier. Briefly, the cells were cultured on glass coverslips, washed, and incubated at 37°C for 1 hour with PBS-HEPES medium containing 5 µM fura-2/AM, 5.4 mM KCl, 2 mM NaPO₄, 0.8 mM MgCl₂, 1.3 mM CaCl₂, 20 mM HEPES-Tris (pH 7.4), and 5 mM glucose. Before analysis, the coverslip was inserted into a thermostat-controlled chamber and then examined with an inverted epifluorescence microscope (Nikon, Tokyo, Japan). After background recording for 40 seconds (20 images), the experiment was initiated by adding different concentrations of JMV449. Fluorescence images were obtained at intervals of 2 seconds, and [Ca²⁺]i concentrations were recorded from the ratio of the fluorescence intensities at 340 and 380 nm on a pixel basis. The dose-response curve for [Ca²⁺]i concentrations was obtained by integrating the area under the curve (measuring calcium transients plotted as a function of time for each field from the addition of the JMV449 until the end of image recording, 200 seconds) and averaging the fluorescence from the whole field of cells chosen. The calcium-stimulation curves were determined for each tested concentration of JMV449 and SR48692. Thapsigargin (1 µM) was used at the end of the experiment to check cell reactivity.

MTS Assay
We also studied keratocyte proliferation using an MTS assay. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) and phenazine methosulfate (PMS) were obtained from Promega (Madison, WI) and Sigma-Aldrich Chimie, respectively. MTS (2 mg/mL; pH 6.5) was dissolved in PBS and filter sterilized. A 5 mM PMS solution was also prepared (in PBS) and filter sterilized. These solutions were stored at −20°C in light-protected containers. To enhance the cellular reduction of MTS, PMS was added
to MTS immediately before use (MTS-PMS ratio: 1:20). A portion of the mixture (150 μL/H9262L) was added to each well. After incubation at 37 °C in a humidified atmosphere with 5% CO2 for 2 hours, 100 μL/H9262L of the supernatant was diluted in 1 mL deionized water. The optical density was measured at 490 nm by means of spectrophotometry. Keratocyte growth was analyzed by means of MTS assay after 72, 96, and 120 hours of culture. Keratocyte proliferation also was analyzed with a hemocytometer and a cell counter (Beckman Coulter, Fullerton, CA).

**Apoptosis Assay**

We also looked for keratocyte apoptosis by nucleus labeling with a fluorescent dye for nuclei after 6 days of culture. Second- and third-passage keratocytes were cultured in THF and treated each day for 5 days with three different concentrations of JMV449 (10⁻⁹, 10⁻⁸, and 10⁻⁷ M) before apoptosis assay. After washing in PBS, the cells were incubated in a solution of 10 μg/mL Hoechst 33342 (Molecular Probes) in PBS for 30 minutes. Hoechst 33342 is a UV fluorescent probe used to visualize apoptosis. It specifically reacts with the DNA at adenine and thymine levels by intercalation after 30 minutes. Fluorescence was analyzed by microplate cold light fluorometry, which allows for fluorometric detection with high sensitivity and specificity. Fluorometry was performed with a microplate cytofluorometer (Fluorolit 1000; Dynex; Cergy-Pontoise, France). Microplate cold light cytofluorometry results were obtained in fluorescence units and were expressed as a percentage of the control. Each JMV449 concentration was tested in six wells, and each experiment was performed in duplicate. To complete these results, the specimens were examined using an epifluorescence microscope (Diaphot TDM; Nikon) with UV filters. Six to 12 photographs of each specimen were taken using the same instrument. Photographs were analyzed by two observers in a blind fashion. The number of apoptotic cells and the total number of cells were counted. Condensed and/or fragmented nuclei appeared highly fluorescent in apoptotic cells.

**Statistical Analysis**

Data were analyzed by analysis of variance and the Wilcoxon rank sum test. Statistical analysis was performed on computer (SPSS for Windows, ver. 9.00; SPSS Science, Chicago, IL).

**RESULTS**

The presence of NT partners was assessed by the detection of mRNA and proteins. Expression of NTR1 mRNA was detected

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932913/)

**FIGURE 1.** Electrophoretic profile of the human NTRs and NT product obtained from RT-PCR. NTR1 (left) lane M: 100-bp DNA ladder; lane 1: first-passage keratocytes; lane 2: second-passage keratocytes; lane 3: MCF-7 cells; lane 4: CHO34 cells, known to express NTR1 (positive control); lane —: negative control. NTR2 (center) lane M: 100-bp DNA ladder; lane 1: third-passage keratocytes; lane 2: second-passage keratocytes; lane 3: first-passage keratocytes; lane 4: second-passage keratocytes (from a second cornea); lane 5: first-passage keratocytes; lane 6: primary culture (from a second cornea); lane 7: hippocampus (positive control); lane 8: negative control; NTR3 (right) lane M: 100-bp DNA ladder; lane 1: first-passage keratocytes; lane 2: second-passage keratocytes; lane 3: third-passage keratocytes; lane 4: MCF-7 cells; lane 5: hippocampus (positive control); lane —: negative control; lane M: 100 bp DNA ladder.

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932913/)

**FIGURE 2.** Western blot analysis of NTR1 protein in human cultured keratocytes. Proteins extracted from two corneas were electrophoresed on 10% SDS-PAGE gel and transferred onto PVDF membrane. The NTR1 receptor protein was revealed with polyclonal anti-NTR1 antibody and compared with the actin protein.
in cultured human keratocytes. A unique PCR product of 637 nucleotides was detected with ethidium bromide after gel electrophoresis (Fig. 1). The size of this band was consistent with the expected fragment size, as determined from the human NTR1 cDNA. The PCR product of 637 bp was sequenced on a shorter sequence and exhibited 100% homology with the sequence from 567 to 1000 of the human NTR1 cDNA. Expression of NTR3 mRNA was detected in cultured human keratocytes, whereas NTR2 and NT were absent (Fig. 1).

A 52-kDa protein corresponding to NTR1 was detected by means of Western blot analysis (Fig. 2). The functionality of NTR1 was checked using calcium imaging to visualize the transduction pathway. When keratocytes were triggered with JMV449, a rapid and large increase in the \([\text{Ca}^{2+}]_i\) concentration was observed after a few seconds. A significant response was detected with 10^{-8} M JMV449 (Fig. 3). The addition of 10^{-6} M SR48692 before stimulation with JMV449 significantly inhibited calcium response (Fig. 3). Thapsigargin (1 \(\mu\)M) was used at the end of the experiment to check cell reactivity.

The sensitivity of the keratocytes to apoptosis was evaluated by fluorescent labeling of nuclei using Hoechst 33342. JMV449 induced a decrease of the fluorescence ratio after treatment, suggesting a reduction of keratocyte apoptosis (Fig. 5). This effect was maximum at 10^{-7} M JMV449. However, 10^{-8} M and 10^{-9} M JMV449 also had significant effects (\(P < 0.005\)). After 120 hours of culture, the percentage of viable cells increased after treatment with JMV449. On the contrary, more intense apoptosis occurred in the control group (Fig. 5).

**DISCUSSION**

We used the RT-PCR technique to show that cultured human corneal keratocytes produced mRNA coding for NTR1. Western blot analysis results confirmed the presence of NTR1 protein. This high-affinity NT receptor is expressed in the brain, intestine, and blood mononuclear cells, but has never been described in the cornea. NTR1 belongs to the superfamily of seven-transmembrane-domain GPCR. Therefore, binding of NT or JMV449 to its high-affinity receptor has been shown to be coupled to \([\text{Ca}^{2+}]_i\) release. The present results of \([\text{Ca}^{2+}]_i\) measurement experiments demonstrated that NTR1 expressed in keratocytes is functional.

Furthermore, we also observed the presence of NTR3 mRNA in human keratocytes. This receptor, which has been recently cloned and for which a possible involvement in intracellular trafficking has been suggested, may also play a role in
FIGURE 4. Keratocyte proliferation studied by MTS assay. Human corneal keratocytes were cultured with various concentrations of JMV449 (10^{-9}, 10^{-8}, and 10^{-7} M). Results of the MTS assay are expressed in optical density, measured by spectrophotometry. Bars: SD (n = 6 for each group). The control group consisted of keratocytes cultured in THF with no JMV449. After 5 days of treatment, JMV449 induced an increase in keratocyte proliferation at concentrations ranging from 10^{-9} to 10^{-7} M. The differences between the proliferative effects of increasing doses of JMV449 were not statistically significant. **Significantly different from the control group by the Wilcoxon rank sum test (P < 0.005).

FIGURE 5. Apoptosis assay. Chromatin condensation was evaluated with Hoechst 33342 stain. (A) A significant concentration-dependent decrease in fluorescence was observed after 5 days of JMV449 treatment, indicating a decrease in chromatin condensation. Bars: SD (n = 6 for each group). **Significantly different from the control group by the Wilcoxon rank sum test (P < 0.005). (B) Condensed and/or fragmented nuclei appeared highly fluorescent in apoptotic cells (arrow, left). The percentages of necrotic keratocytes were significantly lower in the 10^{-7} M JMV449 group (right) than in the control group (left; P < 0.005). Magnification, ×200.
corneal physiology. However, further experiments using specific NTR3 agonist and antibodies, which are actually unavailable, should be conducted, to demonstrate NTR3 expression and functionality.

In contrast, the low-affinity receptor NTR2 was absent. The absence of effect of SR48692, a NTR1 antagonist and a partial NTR2 agonist, on [Ca²⁺]i mobilization, confirms the absence of NTR2.²⁸

We observed a proliferative effect of JMV449 on keratocyte proliferation, whatever the concentration used (10⁻⁹ M - 10⁻⁷ M). In the same culture conditions, we observed that keratocyte apoptosis was significantly decreased by addition of JMV449. It may be suggested that by decreasing keratocyte apoptosis, JMV449 could thus increase cell growth. The effect of JMV449 on keratocyte growth is probably related to NTR1. However, it has recently been shown that NT can stimulate the growth of CHO cells stably transfected with the NTR3.³⁵ Thus, we cannot exclude that NTR3 may partially mediate the effect of JMV449 on keratocyte proliferation.

The present results provide evidence of the existence of a functional NTR1 in corneal keratocytes. They also suggest that NT exerts a trophic effect on keratocytes. Such effects have been described in peripheral tissues containing neurotensinergic innervation. NT stimulates growth of numerous gastrointestinal tissues in vivo, including those of the pancreas, gastric antrum, small bowel, and colon, as well as many cancer cell lines in vitro, such as pancreatic and small-cell lung cancer cells.¹¹,²⁰ The molecular mechanisms of this trophic effect remain largely undefined. However, it has recently been shown that mitogen-activated protein kinases (MAPKs) translocate to the nucleus and induce transcription factors such as the AP-1 and c-Fos in response to NT.³⁵ The question of whether NT is trophic for human cornea in normal and pathologic conditions has to be determined.

The absence of endogenous NT in corneal keratocytes suggests that NT can be present in corneal nerves or released by nearby tissue (i.e., ciliary epithelium or lacrimal gland). NT has never been observed in corneal nerves. However, NT-containing cell bodies and fibers were found in the trigeminal nuclear complex of many animal species—³⁸–⁴⁰ and in the spinal trigeminal nucleus in the human brain.⁴¹ NT peptide immunoreactivity has been found at very low levels (1 ng/g of tissue) in pooled samples of spinal and trigeminal ganglia of the cat.²⁰ Moreover, NT-immunoreactive dorsal root ganglia terminals of the rat spinal cord have been found to contact directly primary afferent terminals.³⁵ These anatomic data suggest the existence of local neurotensin circuits for the control of trophic functions and for the transmission and modulation of sensory information. However, these actions remain to be elucidated, and further experiments are needed to localize NT in the corneal nerve endings.

Genes for NT and its receptor have been recently found to be expressed in the human ocular ciliary epithelium (OCE) and in a human cell line derived from the ciliary pigmented epithelium.¹⁷ NT could thus be secreted in aqueous humor by the OCE and may reach the cornea (paracrine loop). It has been shown that NT peptide is rapidly degraded after its secretion in the aqueous humor, where numerous proteases have been identified.⁴³ However, the possibility that NT might bind to a protein and be carried to a target tissue cannot be ruled out.⁴⁴ On the contrary, clear evidence exists that networks of peptidergic nerves are present within the lacrimal gland and can play a significant role in the modulation of lacrimal protein and fluid secretion. NT has been identified in neurons located in the periclarinar regions, close to the basal surfaces of acinar cells in the camel lacrimal gland.⁴⁵ We can thus hypothesize that NT can be synthesized and secreted by lacrimal gland and released in the tears before reaching the cornea.

In conclusion, we have shown the expression of NTR1 and NTR3 in human corneal keratocytes. JMV449 significantly increased cell proliferation and decreased apoptosis, probably through NTR1. We thus suggest that NT may exert trophic effects on human corneal keratocytes.

References


