Vasoactive Intestinal Peptide Stimulation of Human Trabecular Meshwork Cell Growth

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Purpose. To demonstrate that vasoactive intestinal peptide (VIP), a 28-amino acid neuropeptide, is a growth factor of human trabecular meshwork (TM) cells in culture and in a corneoscleral explant organ culture treated with laser trabeculoplasty (LTP).

Methods. Proliferating human TM cells in cell cultures were incubated with VIP for 20 hours, followed by total cell number determination, using a Coulter counter. The percentage of proliferating TM cells was assessed, using an antibody against the proliferating cell nuclear antigen (PCNA). To test the growth effect of VIP on TM cells in situ, corneoscleral explants in organ cultures were first treated with argon LTP to initiate TM-cell proliferation and then were exposed to VIP for 48 hours. The mitotic TM cells were demonstrated immunocytochemically, using anti-PCNA in paraffin sections of the explants; and the total number of TM cells was determined after paraffin sections were counterstained by hematoxylin.

Results: Vasoactive intestinal peptide dose-dependently stimulated the proliferation of TM cells in cell culture. Treatment with 5 × 10^{-10} M VIP resulted in a maximal increase of 40% in cell number. The effect of VIP was blocked by a VIP antagonist. The number of PCNA-stained TM cells and the total cell number in the TM in LTP-treated corneoscleral explants were increased by VIP.

Conclusions. Exogenously applied VIP stimulated the proliferation of human TM cells in subconfluent cultures and in LTP-treated corneoscleral explants. In that LTP has been shown to increase the number of TM cells in situ, the growth stimulatory effect of VIP may help enhance this therapy. Invest Ophthalmol Vis Sci. 1997;38:2781-2789.

Glaucoma is a blinding disease mainly affecting people more than 40 years old.1,2 Primary open-angle glaucoma is usually associated with elevated intraocular pressure produced by decreased aqueous flow. In spite of intensive studies, the pathogenesis of the disease is not known. The trabecular meshwork (TM), a tissue believed to be involved in the regulation of the aqueous humor outflow, has been the target of many investigations. A decrease in cellularity in the TM correlates with the aging process and the glaucomatous condition of the eyes.3,4

In vivo, most TM cells are postmitotic. However, measurement of thymidine incorporation has yielded results in several studies that suggest that a small number of cells are dividing.5-7 Whether this basal TM-cell mitosis plays a role in maintaining the TM-cell population size in young and healthy TM remains unknown. Our working hypothesis is that an increase in the number of TM cells would be of benefit in the treatment of glaucoma. A common procedure used to treat primary open-angle glaucoma after failure of antiglaucoma drug therapy8-10 is laser trabeculoplasty (LTP), which consists of the application of small focal laser burns to the TM. Although the mechanism by which LTP lowers the intraocular pressure in treated eyes is unknown, LTP increases cell mitosis of TM cells in animal models.6,7 Using human corneoscleral explant organ cultures, investigators have demon-
strated that after LTP treatment, there is an immediate decrease in TM-cell density, followed by increased division of the TM cells and repopulation of the meshwork.\textsuperscript{11,15} The current study has focused on the potential of a growth factor for enhancing the effect of LTP on TM-cell proliferation.

Various growth factors and cytokines are well known for their capacities to stimulate cell proliferation.\textsuperscript{13,14} In the current study, VIP was investigated as a potential growth factor for the stimulation of the growth of proliferating human TM cells, in cell cultures and in situ, in corneoscleral explant organ cultures that have been treated with LTP.

Vasoactive intestinal peptide, a 28-amino-acid neuropeptide, has been found in the aqueous humor,\textsuperscript{15} which continuously bathes the TM in vivo, as well as within the TM.\textsuperscript{16} In addition to its many neuro-modulatory functions, VIP is a regulator of growth and differentiation in a variety of cultured cells, including astrocytes,\textsuperscript{17-20} the retinal pigment epithelium,\textsuperscript{21} keratinocytes,\textsuperscript{22} and smooth muscle cells.\textsuperscript{23} In a homogeneous cell population of cultured chick retinal pigment epithelium, we have shown that VIP stimulates cell proliferation and the synthesis of melanin, a unique differentiation marker of the retinal pigment epithelium.\textsuperscript{21} Recent study results have also shown that VIP exerts a dramatic growth effect on early, postimplantation, whole embryos in culture, where cell proliferation is accompanied by cell differentiation.\textsuperscript{24} In addition to its mitogenic effects on the proliferating cells, VIP promotes the survival of the differentiated cells. For example, VIP not only increases cell proliferation and differentiation, but also promotes the survival of cultured sympathetic neuroblasts.\textsuperscript{25} It also promotes neuronal survival of cultured neurons from the spinal cord,\textsuperscript{26} cerebral cortex,\textsuperscript{27} and hippocampus\textsuperscript{28} through glia-derived factors.

We have previously demonstrated that VIP stimulates the production of cyclic adenosine monophosphate (cAMP) in confluent cultured monkey TM cells in a dose- and time-dependent manner.\textsuperscript{29} In the current study, we demonstrated that VIP stimulated the proliferation of human TM cells in cell cultures and in LTP-treated corneoscleral explants. Our results suggest that VIP may enhance the effect of LTP on TM repopulation with newly propagated TM cells in LTP-treated eyes.

**MATERIALS AND METHODS**

**Cell Culture**

Normal human eyes, obtained from donors 6, 18, 23, 30, and 31 years of age less than 24 hours after death, were received from the Illinois Eye Bank, Chicago. The procedures for culturing TM cells from mamma-

**TREATMENT OF CULTURED TRABECULAR MESHWORK CELLS WITH VASOACTIVE INTESTINAL PEPTIDE**

Three hours after seeding, the medium was removed and replaced with fresh growth medium. Sets of 6 wells in the 24-well plates (or three 35-mm dishes) were added with 10 (or 20) ml sterile water or of VIP to achieve the final VIP concentrations. The specificity of the VIP effect was demonstrated by inclusion of a VIP antagonist, a VIP hybrid (Bachem, Torrance, CA) synthesized by replacing the 1 to 6 amino acids of the native VIP with segments from neurtensin.\textsuperscript{34} The VIP hybrid antagonizes the effect of VIP in stimulating mitosis and proliferation.\textsuperscript{24,35}

**CELL COUNT IN CELL CULTURES**

Cell number in each well was determined 20 hours after addition of VIP. After removal of the medium, cultures were washed three times with 2 ml phosphate-buffered saline (PBS), once with 0.5 ml trypsin solution (0.25%, Gibco, Grand Island, NY), and incubated with 0.1 ml fresh trypsin solution at 37°C for 5 to 10 minutes. The cells were scraped into 1 ml of PBS, and the number in each well was counted with a Coulter counter. The degree of stimulation was calculated by dividing the number of cells in each of the wells with the averaged cell numbers in the control wells within the same experiment.

**PROLIFERATING CELL NUCLEAR ANTIGEN IMMOStAINING OF TRABECULAR MESHWORK CELL CULTURES**

The cultures were immunostained\textsuperscript{36} with a monoclonal antibody against PCNA (PC10, Dako, Carpinteria, CA) for proliferating TM cells. Briefly, cultures were washed three times for 5 minutes, each time with 2 ml PBS, and were fixed in 600 μl 4% paraformaldehyde in PBS for 10 minutes at 4°C. After washing the cultures with 600 μl of PBS twice for 5 minutes, cells were permeabilized with 600 μl methanol (−20°C), incubated for six minutes at room temperature, and incubated for 1 hour with 1% bovine serum albumin in PBS to block the nonspecific binding sites. The immunostaining was performed with a kit (Vectastain
Eric A. Brandt, MD
With support from the Ophthalmic Research Institute (University of California, San Diego) and the National Eye Institute (National Institutes of Health).


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The results of this study were presented at the 2005 and 2007 annual meetings of the American Academy of Ophthalmology.

We thank M. Yeh, MD, and R. Ilsley, MD, for their assistance in conducting the study; the US Food and Drug Administration, Rockville, Maryland, for granting the Investigational New Drug application; and all the participating physicians, nurses, technicians, and research coordinators for their hard work.

The duration of treatment effect on IOP was tested using the Cox proportional hazards model which included the study eye as a random effect. The proportionality assumption was confirmed with log-log plots.

The modified Becker scale was used for grading the severity of ocular complications.

The study was performed at the following sites: the University of California, San Diego, and the Retina Research Center, San Diego, California; the Wills Eye Hospital, Philadelphia, Pennsylvania; Wills Eye Institute, Philadelphia, Pennsylvania; the Wilmer Eye Institute, Baltimore, Maryland; the New York University School of Medicine, New York, New York; the University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; and the University of Iowa, Iowa City, Iowa.

The Ocular Hypertension Treatment Study Group is a collaborative effort of the following: The ocular hypertension treatment study group, sponsored by the National Eye Institute, National Institutes of Health, Bethesda, Maryland; the Retina Research Center, San Diego, California; the Wills Eye Hospital, Philadelphia, Pennsylvania; Wills Eye Institute, Philadelphia, Pennsylvania; the Wilmer Eye Institute, Baltimore, Maryland; the New York University School of Medicine, New York, New York; the University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; and the University of Iowa, Iowa City, Iowa.

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RESULTS
Vasoactive Intestinal Peptide Stimulation of Growth and Proliferation of Trabecular Meshwork Cells in Culture
As was shown in Figure 1, VIP dose-dependently stimulated the proliferation of TM cells in culture (P < 0.05, ANOVA). Optimal stimulation of proliferation was observed at 5 X 10^-10 M VIP. Treatment with 5 X 10^-10 M VIP for 20 hours increased the number of cells to 139 ± 6% of that in the untreated cultures (Fig. 1). The results shown in Figure 1 were averaged from five separate experiments, each using a cell line derived from each of the five donors (Material and Methods). All five TM-cell lines studied demonstrated...
Log [VIP] (M)

**FIGURE 1.** Vasoactive intestinal peptide-stimulated proliferation of cultured human trabecular meshwork cells in a concentration-dependent manner. At $5 \times 10^{-10}$ M, vasoactive intestinal peptide stimulated cell proliferation to 139% of the control cultures in 20 hours. Bar represents mean values ± SEM; analysis of variance showed that there was a statistically significant difference among treatment groups ($P < 0.05$). The degrees of stimulation (Materials and Methods) in cultures treated with $10^{-10}, 5 \times 10^{-10}, 10^{-9},$ and $5 \times 10^{-8}$ M were significantly different from those without vasoactive intestinal peptide treatment at the level of $P < 0.05$, whereas cultures treated with $5 \times 10^{-11}$ M and $10^{-8}$ M vasoactive intestinal peptide were not significantly different from those without vasoactive intestinal peptide treatment.

responsiveness to VIP modulation. Table 1 showed results from representative experiments of $5 \times 10^{-10}$ M VIP-stimulated proliferation of each of the five TM-cell lines.

The stimulatory effect of VIP on proliferation was blocked by an antagonist of VIP, a hybrid peptide synthesized by replacing the 1 to 6 amino acids of the native VIP with segments from neurotensin (Fig. 2; $P < 0.05$; ANOVA). In the presence of $5 \times 10^{-10}$ M VIP, $10^{-7}$ M VIP antagonist decreased the TM-cell number by 31% from $10^5 \pm 7 \times 10^3$ cells/well to $72 \pm 7 \times 10^3$ cells/well.

The effect of VIP on cultured TM-cell proliferation was also observed in the serum-free medium. As shown in Table 2, in the absence of serum, $5 \times 10^{-10}$ M VIP significantly increased the number of TM cells from the control level of $16.5 \pm 1 \times 10^3$ cells/well to

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<th>TABLE 1. Growth Effect of Vasoactive Intestinal Protein on Trabecular Meshwork Cell Lines Derived From Five Donors</th>
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<tr>
<td><strong>Cell Line</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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VIP = vasoactive intestinal protein.

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<th>TABLE 2. Cultured Trabecular Meshwork Cell Proliferation in Serum-Free Medium: Effect of Vasoactive Intestinal Protein and Serum</th>
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<tr>
<td><strong>Agent</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>$5 \times 10^{-10}$ M VIP</td>
</tr>
<tr>
<td>5% fetal calf serum</td>
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Analysis indicated that the numbers of trabecular meshwork cells in wells treated with $5 \times 10^{-10}$ M vasoactive intestinal peptide (VIP) and that with 5% fetal calf serum were significantly different from those in the serum-free medium at the levels of $P = 0.02$ and $P = 0.004$, respectively.
Vasoactive intestinal peptide treatment after laser trabeculoplasty of corneoscleral explants stimulated in situ trabecular meshwork cell proliferation

After applying 50 laser burns to initiate TM-cell division, the corneoscleral explants were incubated in culture medium in the absence or presence of VIP for 48 hours. The proliferating TM cells in the LTP-treated corneoscleral explants were identified by immunocytochemical studies of the paraffin sections of the explants using anti-PCNA. As shown in Figure 4, the presence of $1 \times 10^{-7}$ M VIP in the incubation medium (Fig. 4B) produced a large increase in the number of anti-PCNA-positive TM cells in the corneoscleral explants, whereas few of the TM cells appeared anti-PCNA-positive in the absence of VIP (Fig. 4A). Sections stained with the mouse IgG at a concentration twice that of IgG in the anti-PCNA were used as negative controls (Fig. 4C). The $5 \times 10^{-10}$ M VIP-treated corneoscleral explants showed fewer anti-PCNA-positive TM cells than those treated with $1 \times 10^{-7}$ M VIP (data not shown). Although Figure 4 depicted results of experiments using one of the 17-year-old donor eyes (OD), it was representative of results in all seven donor eyes (see Materials and Methods).

The number of nuclei in the TM area was counted in each of the hematoxylin-stained sections of corneoscleral explants derived from the four eyes of the 17-year-old and the 39-year-old donors (see Materials and Methods). Table 3 showed results obtained from corneoscleral explant paraffin sections derived from one of the eyes (OD) of the 39-year-old donor. The number of cells (nuclei) in the TM was increased by VIP in a dose-dependent manner ($P < 0.05$, ANOVA). Without the VIP treatment after LTP, the average number of cells in one TM section was $64 \pm 69$ ($n = 28$ sections). Treatment with $5 \times 10^{-10}$ and $1 \times 10^{-7}$ M VIP increased the averaged number of cells (nuclei) in one TM section to $82 \pm 3$ ($n = 43$ sections) and $115 \pm 5$ ($n = 44$ sections), respectively. Similar results were observed in the remaining three eyes examined (data not shown).

**DISCUSSION**

The growth-promoting effect of VIP has been demonstrated in numerous culture systems, but not in an organ that is populated with differentiated cells. We are the first to demonstrate the mitogenic effect of VIP on TM cells in situ after wounding by laser burns (Fig. 4). Demonstration of VIP’s effect on proliferation (Figs. 1, 2, 3) of human TM-cell cultures lends support to the direct modulatory role of VIP. Unlike explants, TM cells in subconfluent culture do not require LTP to initiate proliferation, and they proliferate even more vigorously with VIP in a dose-dependent manner (Figs. 1, 3).

In TM-cell cultures, the optimal concentration of VIP in stimulating the proliferation was $5 \times 10^{-10}$ M (Figs. 1, 3), a concentration at which VIP was ineffective in stimulating cAMP production in confluent TM-cell cultures. Vasoactive intestinal peptide stimul-
FIGURE 4. Vasoactive intestinal peptide treatment after laser trabeculoplasty stimulated trabecular meshwork cell proliferation in corneoscleral explants. Paraffin sections of explants treated with (A) 0 M vasoactive intestinal peptide or (B, C) $1 \times 10^{-7}$ M vasoactive intestinal peptide after laser trabeculoplasty were stained with (A, B) anti-PCNA to show the proliferative cells or (C) mouse IgG as negative controls. Round pigment granules were present in the trabecular meshwork in all three sections. Magnification: $\times 400$. PCNA = proliferating cell nuclear antigen.

tion of proliferation of cultured cells typically occurs at VIP concentrations several folds of magnitude lower than that required for the stimulation of cAMP production. The effectiveness of VIP in stimulating the TM-cell growth in cell culture is comparable to that of the basic fibroblast growth factor (bFGF). In results of our preliminary studies, VIP and bFGF (both at $5 \times 10^{-10}$ M) stimulated the growth of TM cells in culture from the basal level of $43.4 \pm 2.4 \times 10^6$ to $67.9 \pm 4.3 \times 10^6$ and $70.8 \pm 10.3 \times 10^6$ cells/well, respectively. Vasoactive intestinal peptide is a true mitogen of the TM cells, in that its effect was also observed in cultures growing in serum-free medium (Table 2). Although the signaling pathways of growth factors (bFGF) whose receptors in the plasma membranes possess intrinsic tyrosine kinase activities have become well defined, the molecules that mediate the VIP growth stimulation of cultured cells remain largely unknown.

The TM-cell cultures were not stimulated effectively to proliferate by VIP at high concentrations ($1 \times 10^{-6}$ M) that are known to stimulate the production
VIP on the Growth of Human Trabecular Meshwork Cells

**TABLE 3. Effect of Vasoactive Intestinal Protein Treatment on the Trabecular Meshwork Cell Population Sizes in Corneoscleral Explants After Laser Trabeculoplasty**

<table>
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<tr>
<th>[VIP] (M)</th>
<th>Cell Number/TM Section</th>
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<tr>
<td>0</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>$5 \times 10^{-10}$</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>113 ± 5</td>
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Data represent the numbers of TM cells (mean ± SEM) in one paraffin section derived from wedges of corneoscleral explants (OD of the 39-year-old donor treated with 0, $5 \times 10^{-10}$ and $1 \times 10^{-7}$ M VIP). The numbers of paraffin sections examined were 28 (0 M), 43 ($5 \times 10^{-10}$ M) and 44 ($1 \times 10^{-7}$ VIP). Six regions in each of the wedges were examined. Statistical analysis (ANOVA) showed that there was a statistically significant difference among the treatment groups ($P < 0.05$). The numbers of TM cells in explants treated with $5 \times 10^{-10}$ M VIP and that with $1 \times 10^{-7}$ M VIP were both different from those in explants without VIP treatment at the level of $P < 0.05$, and the difference between the two groups was different at the level of $P < 0.05$.

VIP = vasoactive intestinal peptide; TM = trabecular meshwork.

of large amounts of intracellular cAMP in confluent TM cultures. The response of human TM-cell cultures to high concentrations of VIP was thus the same as that observed in cultured chick retinal pigment epithelium. As in numerous other systems, high concentrations of VIP and cAMP have been shown to stimulate the differentiation (melanin genesis) of the retinal pigment epithelial cells. The role of cAMP in the promotion of differentiation in other cells has also been well established. It remains to be investigated whether the biogenesis of any of the differentiation markers was stimulated by VIP at high concentrations in TM-cell cultures. Nevertheless, it was not surprising to find that at $10^{-6}$ M, VIP did not stimulate the growth in cell numbers (Fig. 1) or the mitosis of cultured TM cells (Fig. 3), because cell proliferation and differentiation are mutually exclusive processes.

In LTP-treated corneoscleral explants, $5 \times 10^{-10}$ M VIP was ineffective in increasing the number of proliferating (data not shown) or the total number of TM cells (nuclei; Table 3), whereas $10^{-7}$ M VIP, which effectively stimulated cAMP production in confluent cultured TM cells, was also effective in stimulating the proliferation of TM cells in the LTP-treated corneoscleral explants (Fig. 4 and Table 3). The requirement for higher concentrations of VIP in the LTP-treated corneoscleral explants than that in cell cultures may have resulted from the TM cells’ inaccessibility to VIP in the explants. Conversely, our results may indicate that the mechanism of VIP stimulation of the proliferating TM cells in LTP-treated corneoscleral explants and that in cell cultures were different.

In the LTP-treated corneoscleral explants, events initiated by treatment with high concentrations of VIP, and therefore increased intracellular cAMP levels, may take precedence over the VIP (at low concentrations) stimulation of TM-cell mitosis. Vasoactive intestinal peptide, through its stimulation of cAMP production, modulates the expression of genes that contain the cAMP-responsive element. One of these genes is the transcription factor c-fos, which can in turn modulate the expression of other genes. In the lacrimal gland, the expression of c-fos is stimulated by VIP. Very recently, it has been reported that in corneoscleral explants, LTP initiates the synthesis of a mitogenic factor and its release into the conditioned medium.

In addition to TM cells, there are other cells present in the corneoscleral explants. Thus, it is probable that cells other than the TM cells play some role in the modulation of TM-cell proliferation after LTP. Corneal endothelial cells in culture synthesize several cytokines including interleukin-1. Vasoactive intestinal peptide, a well-known secretagogue, stimulates the secretion of interleukin-1 and interleukin-6 in cultured glial cells. The mitogenic activities of cytokines are well documented. Results of our preliminary studies have shown that VIP-stimulated intracellular cAMP production in cultured rabbit corneal endothelial cells in dose- and time-dependent manners (Koh and Yue, unpublished data). Thus VIP treatment of the corneoscleral explants may have led to increased levels of secretions of cytokines in the conditioned medium of explants and may have indirectly stimulated the proliferation of those TM cells that have regained their proliferative potential after LTP.

We hypothesize that VIP is a human TM-cell growth factor capable of stimulating the proliferation of mitotic TM cells after LTP. The mechanism by which VIP exerts its growth effects on human TM cells after LTP remains to be investigated. Although the link between LTP-induced TM-cell proliferation (as observed in animal studies and in human corneoscleral explants) and the beneficial effect of LTP in lowering the intraocular pressure has not been established, repopulating the TM with newly propagated cells in glaucomatous eyes with significant loss of TM cells may well lead to the restoration of normal TM functions, including regulation of intraocular pressure. Finally, our results are the first to demonstrate the potential of a growth factor to enhance the effect of LTP on the stimulation of TM-cell proliferation.

**Key Words**

argon laser trabeculoplasty, cell proliferation, proliferating cell nuclear antigen, trabecular meshwork cells, vasoactive intestinal peptide

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