Somatostatin Is an Immunosuppressive Factor in Aqueous Humor

Andrew W. Taylor¹,² and David G. Yee¹

PURPOSE. To detect the presence of somatostatin (SOM) in normal aqueous humor and to characterize its immunosuppressive activity.

METHODS. Fresh rabbit aqueous humor was assayed for SOM by competitive ELISA. Primed T cells stimulated through their T-cell receptor (TCR) were treated with SOM at concentrations that ranged the level of SOM found in normal aqueous humor. The T cells were assayed for proliferation, lymphokine production, and immunosuppressive activity.

RESULTS. Normal rabbit aqueous humor contained 196 ± 45 pg/mL (10⁻¹⁰ M) of SOM. At concentrations between 10 and 500 pg/mL, SOM suppressed IFN-γ production by TCR-stimulated primed T cells in culture. Frozen and thawed aqueous humor depleted of SOM no longer suppressed IFN-γ production by the TCR-stimulated primed T cells. SOM induced TGF-β but not IL-4 production, nor did it suppress proliferation by TCR-stimulated primed T cells. The SOM-treated T cells functioned as regulatory T cells, and this regulatory activity was neutralized by anti-α-MSH antibodies. Furthermore, SOM induced α-MSH production by the TCR-stimulated primed T cells.

CONCLUSIONS. SOM is present in aqueous humor and contributes to the immunosuppressive activity of aqueous humor. Moreover, SOM induces the production of the potent immunomodulating factor α-MSH by TCR-stimulated primed T cells through which the SOM-treated T cells suppress other T cells. Thus, SOM can contribute to the ocular immunosuppressive microenvironment by promoting the production of immunosuppressive cytokines and inducing the activation of regulatory T cells. (Invest Ophthalmol Vis Sci. 2005;44:2644–2649) DOI: 10.1167/iovs.02-1216

For the past decade, it has become more evident that there are several mechanisms of immunosuppression and immunoregulation used by the ocular microenvironment to establish and maintain immune privilege.¹,² The result of such mechanisms is an extreme example of regional immunity that prevents the activation of primed T cells that mediate inflammation. Part of the mechanisms of immunosuppression involve soluble factors found in normal aqueous humor. Some of the immunosuppressive factors in aqueous humor are a family of cytokines originally defined by their association with the nervous system.³ These cytokines target and suppress the inflammatory activity of T cells and antigen-presenting cells.³ The cytokines are α-melanocyte stimulating hormone (α-MSH), va

soactive intestinal peptide (VIP), and calcitonin gene-related peptide (CGRP).³⁻⁶ We have recently discovered that, besides suppressing the inflammatory activity of primed T cells, α-MSH mediates the induction of CD25⁺ CD4⁺ regulatory T (Treg) cells.⁷⁻⁹ Collectively, these factors, along with latent transforming growth factor (TGF)-β2 provide the ocular microenvironment with the means to suppress local activation of immunemediated inflammation and a possible mechanism to regionally induce Treg cells to suppress immunity against ocular antigens.

The main sources of the neurologically associated cytokines are sympathetic and sensory neurons terminating in the tissues of the anterior chamber.¹⁰ There is very little evidence that any of these cytokines are synthesized by neural tissues in the eye except somatostatin (SOM).¹¹⁻¹⁷ Protein and messenger RNA for pro-SOM are in amacrine cells and some cells of the inner plexiform layer of the retina. Receptors for SOM are found on peripheral blood T cells, and a subset of granulomatous T lymphocytes.¹⁵⁻¹⁸⁻²¹ SOM suppresses IFN-γ production by mitogen-stimulated T cells.¹⁸⁻¹⁹⁻²²⁻²³ In addition, SOM mediates a change in the pattern of lymphokines produced by differentiated T cells.²⁴

Because we have found several neurologically associated immunosuppressive cytokines in aqueous humor,⁴⁻⁶ we examined normal rabbit aqueous humor for the possible presence of SOM. We also assessed the ability of SOM at its physiologica ocular concentration to regulate the activity of primed T cells. We not only found biologically active SOM in normal aqueous humor, but also found that SOM can contribute to the immunosuppressive activity of aqueous humor.

METHODS

Reagents and Laboratory Animals

The anti-mouse IFN-γ and anti-mouse IL-4 ELISA antibody pairs and the T-cell receptor (TCR) CD3ε antibody 2C11 were from BD Pharmacia (San Diego, CA). Recombinant IFN-γ and IL-4 for standards in the ELISA were obtained from R&D Systems (Minneapolis, MN). Synthesized and biotinylated SOM and α-MSH were obtained from Peninsula Laboratories (Belmont, CA), along with purified antibodies to SOM and α-MSH. The anti-SOM antibody is a rabbit IgG reactive to SOM, SOM25, and SOM28, but not to other SOM analogues, pro-SOM, or other neuropeptides. The anti-α-MSH is the same rabbit IgG antibody we have used before³ and is specifically reactive to α-MSH, but not to adrenocorticotropic hormone. Laboratory animals used in the experiments were New Zealand White rabbits (Millbrook Breeding Laboratories, Amherst, MA) and BALB/c mice (institutional breeding program). All laboratory animal use was approved by the Schepens Institutional Animal Care and Use Committee based on the U.S. Public Health Service Guide for the Care and Use of Laboratory Animals and complied with the regulations (9 CFR, Subchapter A) issued by the U.S. Department of Agriculture (USDA) under the Animal Welfare Act. Animal use also conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Aqueous Humor Collection

Aqueous humor was obtained from normal New Zealand White rabbits by passive drainage of the anterior chamber.⁵,⁶ The rabbits were
anesthetized with a mixture of ketamine and xylazine intramuscularly, and the aqueous humor was obtained by limbal paracentesis with a 27-gauge winged infusion set (Surflo; Fisher Scientific, Pittsburgh, PA). Aqueous humor was allowed to flow passively into a siliconized microcentrifuge tube (Fisher Scientific).

**ELISA to Quantify SOM**

The concentration of SOM in fresh normal rabbit aqueous humor was measured by a competitive ELISA method similar to the ELISA we developed for α-MSH, VIP, and CRF. *Somatostatin* aqueous humor samples were mixed with 2 ng/mL biotinylated SOM and added to the wells of a 96-well flat-bottomed tissue culture plate (Corning Costar, Corning, NY) that was first coated overnight with a 1:500 dilution of anti-Rb IgG (Sigma Chemical Co., St. Louis, MO), blocked, and incubated with a 1:400 dilution of anti-SOM. For a standard curve, known amounts of SOM protein (10–0.003 ng/mL) in Dulbecco’s PBS were mixed with the biotinylated-SOM. The buffer we used to block, wash the wells, and dilute the antibodies was 1% BSA (Sigma) in 0.1 M PBS (PBS-BSA). After a 2-hour incubation at room temperature, the wells were washed and streptavidin-β-galactosidase (Gibco BRL, Gaithersburg, MD), diluted 1:1000, was added to the wells. The plate was incubated for 30 minutes at room temperature and washed, and the substrate chlorogen-b-O-galactosidase (Gibco BRL) was added to the wells. The optical density of the color change was read 1 hour later with a microplate spectrophotometer (μQuant; Bio-Tek, Winooski, VT). The concentration of SOM in the aqueous humor was calculated by using the sample’s optical density (OD) in an equation fitting the polynomial regression of known SOM concentrations to their OD. The SOM competitive ELISA was sensitive down to 3 pg/mL. For quantifying α-MSH in the T-cell culture supernatants, we used competitive ELISA methods identical with the ones that we had developed and used, with a limit of detection of 1 pg/mL.

**Lymph Node T-Cell Assay for Cytokine Production and Proliferation**

We evaluated the effects of SOM on primed lymph node T cells, with a T-cell culture assay previously reported. Briefly, we collected cells from mouse lymph nodes that drain tissue sites injected with Freund’s adjuvant containing 10 mg/mL *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI). T cells were enriched using a T-cell column (R&D Systems). This method obtained 99% CD3+ cells by flow cytometry analysis. The T cells (4 × 10⁵ cells) were added to the wells of a 96-well, round-bottomed plate (Corning Costar) along with SOM and 1 μg/mL anti-CD3ε antibody (145-2C11; BD Pharmingen, San Diego, CA) in serum-free medium. The concentration of anti-CD3ε antibody was optimized for IFN-γ production and proliferation by primed lymph node T cells. At this concentration, the antibody did not stimulate naive lymph node T cells, which required 10 times more antibody to be stimulated. The serum-free culture medium was RPMI 1640, 0.1% BSA solution (Sigma, St. Louis, MO), and a 1:500 dilution of ITS+1 solution (Sigma). The cultures were incubated for 48 hours at 37°C in 5% CO₂, and the supernatants were assayed for lymphokines using sandwich ELISA specific for IFN-γ or IL-4. The supernatant was also assayed for TGF-β using the standard Mv1Lu (CCL-64 cell line; from ATCC, Manassas, VA) bioassay for TGF-β. The lymphokine assays are described in detail in the next section. For the proliferation assay, the T-cell cultures were initially incubated for 24 hours, 20 μL of 50 μCi/mL [³H]thymidine (REN, Boston, MA) was added to the wells, and the cultures were incubated for an additional 24 hours. The cells were collected onto filter paper by a plate harvester (Tomtec, Hamden, CT), and radiolabel was measured with a liquid scintillation counter (Beta-plate; Wallac, Gaithersburg, MD).

**Lymphokine Assays**

Cytokine production was assayed for IFN-γ, IL-4, and TGF-β in the culture supernatant of stimulated T cells incubated for 48 hours as we previously reported. The concentrations of IFN-γ and IL-4 in the supernatant were measured by sandwich ELISA, and TGF-β was measured by bioassay. In brief, culture supernatants or known concentrations of recombinant cytokine for a standard curve were added to the wells of a 96-well microtiter plate, coated with capturing monoclonal antibody to the specific cytokine being assayed, and blocked. After a 3-hour incubation at room temperature, the plate was washed and biotinylated detecting antibody to the cytokine was added and incubated for 1 hour. This was followed by a 30-minute incubation of streptavidin-β-galactosidase (Gibco BRL) and a 1-hour incubation of the substrate chlorogen-b-D-galactoside (Calbiochem, La Jolla, CA). The optical density of the color change was read on a standard ELISA plate reader at a wavelength of 574 nm, and, based on the optical density of the known concentrations of recombinant lymphokine, a standard curve was calculated. The sample’s optical density was applied to the standard curve, and the concentration of the lymphokine in the sample was calculated. The limit of detection for IFN-γ was 10 pg/mL and for IL-4 was 20 pg/mL.

To assay for TGF-β, the cultures were incubated for 48 hours and the culture supernatants were collected and assayed for TGF-β by the Mv1Lu cell bioassay as described previously. Culture supernatants were treated with acid for 30 minutes and the acid was neutralized. The transiently acidified samples were diluted 1:8 in 0.5% FBS EMEM and added to cultures of 1 × 10⁵ Mv1Lu cells in a flat-bottomed 96-well culture plate. The cultures were incubated for 20 hours at 37°C in 5% CO₂ and [³H]thymidine (0.5 μCi/well) was added. The cultures were further incubated for an additional 4 hours. Incorporated [³H]thymidine was measured by scintillation counting. TGF-β concentrations of the samples were calculated by the suppression of Mv1Lu cell proliferation in comparison with the suppression in proliferation by known amounts of pure TGF-β1 (R&D Systems). This bioassay had a limit of 10 pg/mL of TGF-β1.

**SOM Absorption from Aqueous Humor**

To neutralize SOM activity in aqueous humor, aqueous humor samples that were previously assayed for SOM and positive for SOM were frozen in siliconized microfuge tubes. The aqueous humor samples were thawed and anti-SOM antibody (40 μg/mL) was added to 25 μL of aqueous humor. For control experiments, instead of the anti-SOM antibody, the same concentration of purified IgG from naive rabbit serum was added to 25 μL of aqueous humor. The samples were incubated at room temperature for 30 minutes, and 10 μL of a 50% solution of protein-G beads (Pierce Biototechnology, Rockford, IL) was added followed by an additional incubation of 30 minutes at room temperature with constant agitation. The samples were centrifuged at 2500g for 10 minutes to pellet the beads. The supernatants (SOM-absorbed aqueous humor) were used to treat primed T cells activated in the T lymphocyte assay. The T-cell culture supernatants were assayed by ELISA for IFN-γ 48 hours later.

**Regulatory T-Cell Assay**

The regulatory T-cell activity was assayed as described in previous publications. The T cells were obtained from primed lymph nodes as described earlier activated with anti-CD3ε antibody and treated with SOM at 3, 30, or 300 pg/mL for 48 hours. These SOM-treated T cells were collected and used as regulatory T cells (SOM-treated T cells) by adding them at 4 × 10⁵ cells/well into secondary cultures already containing fresh anti-CD3ε-stimulated T cells (4 × 10⁵ cells/well) from primed lymph nodes. We also added to some of these secondary cultures anti-TGF-β antibodies (R&D Systems) or anti-α-MSH antibodies (Peninsula Laboratories, Belmont, CA). The cultures were incubated for 48 hours, and the culture supernatant was assayed for IFN-γ by sandwich ELISA. Suppression of IFN-γ production was an indication that the added SOM-treated T cells mediated regulatory activity.
TCR-stimulated primed T cells (Table 1). Therefore, the SOM in culture supernatant was assayed for IFN-γ antibody (TCR-stimulated). After 48 hours of incubation, the added aqueous humor samples by ELISA for SOM and contained the highest concentrations of SOM. Assaying the thawed aqueous humor found that SOM protein remained, whereas, SOM was absorbed from the thawed aqueous humor, and the longer detected in the aqueous humor (data not shown). The culture supernatant was assayed for IFN-γ production (Fig. 1). We found significant suppression of IFN-γ production with increasing concentrations of SOM added to the T-cell cultures. At its ocular physiological concentration, SOM suppressed IFN-γ production.

To detect SOM activity in aqueous humor, we absorbed SOM from frozen and thawed samples of aqueous humor. Because α-MSH is the most potent immunosuppressive and immunomodulating factor in aqueous humor to suppress IFN-γ production,4,5,7,9,25,27,28 we subjected the aqueous humor to a cycle of freezing and thawing to destroy the α-MSH activity. This is why fresh aqueous humor has been assayed in the past.4,5,25,26 We froze the aqueous humor samples that contained the highest concentrations of SOM. Assaying the thawed aqueous humor samples by ELISA for SOM and α-MSH, we found that SOM protein remained, whereas, α-MSH was no longer detected in the aqueous humor (data not shown). The SOM was absorbed from the thawed aqueous humor, and the absorbed aqueous humor was added to cultures of TCR-stimulated primed T cells. The culture supernatant was assayed for IFN-γ 48 hours later. Two examples of thawed aqueous humor with the SOM removed from it demonstrated that without SOM the aqueous humor could not suppress IFN-γ production by TCR-stimulated primed T cells (Table 1). Therefore, the SOM in

### RESULTS

#### SOM in Normal Aqueous Humor

We examined aqueous humor samples from 18 normal rabbit eyes. A competitive ELISA designed in a similar manner for quantifying other neuropeptides in aqueous humor was used to assay the aqueous humor samples.4,6 In rabbit aqueous humor, we detected 192 ± 46 pg/mL (10^-10 M) SOM. This concentration of SOM was in the range of activity reported to suppress IFN-γ production by activated T cells. To see whether SOM at its ocular physiological concentration can suppress IFN-γ production by primed lymph node T cells, SOM was added to cultures of primed T cells stimulated with anti-CD3 antibody (TCR-stimulated). After 48 hours of incubation, the culture supernatant was assayed for IFN-γ production (Fig. 1). We found significant suppression of IFN-γ production with increasing concentrations of SOM added to the T-cell cultures. At its ocular physiological concentration, SOM suppressed IFN-γ production.

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### Table 1. SOM-Induced Suppression of IFN-γ Production in Aqueous Humor by TCR-Stimulated Primed T Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>IFN-γ (pg/mL)</th>
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<tr>
<td></td>
<td>SOM Absorbed</td>
</tr>
<tr>
<td>AqH 1</td>
<td>308 ± 31</td>
</tr>
<tr>
<td>AqH 2</td>
<td>454 ± 46</td>
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<tr>
<td>Positive control</td>
<td>—</td>
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<tr>
<td>Negative control</td>
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AqH 1 and 2, aqueous humor samples 1 and 2.
* The data are the average concentration (pg/mL ± SEM) of IFN-γ in the culture supernatant of TCR-stimulated primed T cells treated with frozen aqueous humor. The aqueous humor was absorbed or not absorbed by SOM.
† These levels of IFN-γ are significantly (P < 0.05) different from IFN-γ levels in positive control cultures.

**FIGURE 1.** SOM suppressed IFN-γ production by TCR-stimulated primed T cells. In culture, primed T cells were stimulated with anti-CD3 antibody and treated with SOM (0–500 pg/mL). After 48 hours of incubation, the culture supernatants were assayed for IFN-γ by ELISA. The unstimulated control had no anti-CD3 antibody and no SOM added to the primed T cell cultures (U). Data are presented as mean nanograms per milliliter ± SEM of results in eight independent experiments. *Significantly different (P ≤ 0.05, unpaired t-test) from the cultures of TCR-stimulated primed T cells with no SOM treatment (†).

**FIGURE 2.** SOM induced TGF-β production by TCR-stimulated primed T cells. As in Figure 1, the primed T cells were stimulated with anti-CD3 antibody and treated with SOM (0–500 pg/mL). The culture supernatants were (A) assayed for TGF-β by bioassay and (B) assayed for IL-4 by ELISA. The assay results are presented as mean nanograms per milliliter ± SEM of results in eight independent experiments. (C) The cultures were assayed for proliferation by scintillation counting after 48 hours of incubation. Data are presented as mean counts per minute (CPM) ± SEM of results in four independent experiments. *Significantly different (P ≤ 0.05, unpaired t-test) from the cultures of TCR-stimulated primed T cells with no SOM treatment (†). The unstimulated control had no anti-CD3 antibody and no SOM added to the primed T cell cultures (U).
aqueous humor can contribute to the regional immunosuppressive activity of aqueous humor.

**Functioning of SOM-Treated Primed T Cells**

Cultures of primed T cells stimulated with anti-CD3 were treated with SOM and assayed 48 hours later for IL-4, TGF-β, and proliferation (Fig. 2). At its range of ocular physiological concentrations of 10 to 300 pg/mL, SOM induced TGF-β production by the TCR-stimulated primed T cells (Fig. 2A). There was no significant presence of IL-4 in the cultures. All the IL-4 concentrations were at the level of sensitivity or below for the IL-4 ELISA (Fig. 2B). In addition, SOM had no effect on TCR-stimulated proliferation by the primed T cells (Fig. 2C).

The primed T cells activated in the presence of SOM produced TGF-β but IL-4 and IL-4 production was suppressed, suggesting that SOM induced the activation of regulatory T cells that we have seen before mediated by whole aqueous humor. To assay for regulatory activity by the SOM-treated T cells, primed T cells were TCR-stimulated and treated with SOM at 3, 30, or 300 pg/mL. After 48 hours of incubation, these T cells were collected and used as regulatory T cells. They were placed into new cultures containing freshly activated primed T cells. The SOM-treated T cells acted as regulatory T cells in that they suppressed IFN-γ production by the other stimulated primed T cells (Fig. 3). The regulatory activity was proportional to the concentration of SOM used to induced the regulatory T cells. To identify the mechanism by which the SOM-induced regulatory T cells suppress the production of IFN-γ by other T cells, neutralizing anti-TGF-β antibody with the regulatory T cells was added to the cultures of freshly activated primed T cells (Fig. 3, E).

**SOM-Induced α-MSH Production by Activated Primed T Cells**

Because the suppressive activity of SOM-induced regulatory T cells was neutralized by anti-α-MSH antibodies, we measured α-MSH production by TCR-stimulated primed T cells treated with SOM. Production of α-MSH by TCR-stimulated T cells was induced by SOM (Fig. 4). TCR-stimulated primed T cells not treated with SOM produced lower amounts of α-MSH than resting primed T cells (Fig. 4). Therefore, SOM induced acti-
uated T cells to produce α-MSH, through which they suppressed the activation of other T cells.

**DISCUSSION**

Similar to other immunosuppressive cytokines in aqueous humor, \(^{6,7,25}\) SOM suppressed IFN-γ production by effector T cells. In addition, SOM mediated the induction of regulatory T cells. These T cells produced TGF-β and α-MSH. Through their production of α-MSH, the SOM-induced regulatory T cells suppressed IFN-γ production by other activated T cells. Our findings suggest that SOM contributes to the normal immunosuppressive microenvironment of the eye.

Because SOM has been clearly demonstrated to be synthesized and released in the retina, \(^{15}\) it is possible for SOM to contribute to immunosuppression in the retina. Although it is known that the retina is an immune-privileged tissue, \(^{65}\) very little is understood about the mechanisms of retinal immunosuppression. The SOM-mediated suppression of IFN-γ and induction of regulatory T cells suggests that similar characteristics of immunosuppression could be present in the retina as is present in the anterior chamber. Therefore, cytokine-mediated immunosuppression may also be a mechanism of retinal immune privilege. In addition, the expression of SOM in the eye may be important in limiting the severity of uveitis. Granulomatous macrophages constitutively produce SOM, which in turn suppresses IFN-γ production by T cells within the granuloma. \(^{16,19,22,35}\) There are several reports describing granuloma formations within the retinas of mice at the peak of autoimmune disease. \(^{51-53}\) Therefore, there exists the possibility that within the uveitic retina the contribution of retinal cell production of SOM along with SOM from granulomatous macrophages could contribute to the characterized resolution of autoimmun uveitis in rodent retinas. Based on our finding that SOM induces regulatory T cells, there may also be an induction of regulatory T cells that promote reestablishment of tolerance to retinal antigens. Regulatory T cells develop after autoimmune uveitis in mice (Taylor AW, unpublished observation, 2002); whether SOM influences this induction of regulatory T cells is to be determined.

Our finding that SOM induces α-MSH production by TCR-stimulated primed T cells suggests that within the ocular microenvironment there is a mechanism for amplifying a cytokine network of immunosuppression. It has been shown that α-MSH induces its own production and receptor expression in monocytes and macrophages. \(^{34,54,55}\) In the presence of SOM, primed T cells activated by antigen-presenting cells would be suppressed in IFN-γ production, whereas their production of α-MSH and TGF-β would be induced. The α-MSH and TGF-β in turn would suppress inflammation \(^{25,27,28,54,56}\) and induce their own production \(^{24,35}\) by the antigen-presenting cells. The further production of α-MSH and TGF-β would enhance immunosuppression in the ocular microenvironment \(^{4,25}\) and further promote the activation of regulatory T cells. \(^{3,4}\) This may also be important for immune homeostasis in the eye. A continuous diffusion of SOM from the retina could promote and maintain constitutive production of α-MSH by immune cells that are resident or have migrated into the ocular microenvironment. In addition, TGF-β levels would be elevated \(^{26,38}\) promoting immune deviation and other immunosuppressive activity in the immune privileged microenvironment. \(^{3,57}\) Therefore, a network of immunosuppressive cytokines could be working within the immune privileged eye.

We have characterized the presence and activity of four immunosuppressive cytokines α-MSH, CGRP, VIP, and now SOM, which are constitutively present in normal aqueous humor. \(^{4-6}\) We know that these cytokines participate in suppressing the induction of inflammatory activity by primed T cells \(^{1-6,25}\) and the activation of innate and IFN-γ-mediated inflammatory responses \(^{8}\) and that they induce the activation of CD4⁺CD25⁺ regulatory T cells. \(^{7,9,26}\) Our new findings imply that an additional mechanism in maintaining immunosuppression in the ocular immune-privileged microenvironment is a potential self-perpetuating network of immunosuppressive cytokines.

**References**


