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Localized retinal neuropeptide regulation of macrophage and microglial cell functionality

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Abstract

The functionality of immune cells is manipulated within the ocular microenvironment to protect the sensitive and non-regenerating light-gathering tissue from the collateral damage of inflammation. This is mediated partly by the constitutive presence of immunomodulating neuropeptides. Treating primary resting macrophages with soluble factors produced by the posterior eye induced co-expression of Arginase1 and NOS2. The neuropeptides alpha-melanocyte stimulating hormone and Neuropeptide Y alternatively activated the macrophages to co-express Arginase1 and NOS2 like myeloid suppressor cells. Similar co-expressing cells were found within healthy, but not in wounded retinas. Therefore, the healthy retina regulates macrophage functionality to the benefit of ocular immune privilege.

Keywords

Alpha-melanocyte stimulating hormone; Alternative macrophage activation; Neuroimmunomodulation; Neuropeptide Y; Ocular immune privilege; Retina

1. Introduction

The ocular microenvironment has adapted several mechanisms of immunomodulation to protect its delicate light gathering tissues from the collateral damage of inflammation. These mechanisms actively manipulate immune cells away from inflammatory pathways, and induce immune regulation and tolerance (Hara et al., 1993; Kitaichi et al., 2005; Sonoda et al., 2005; Sugita et al., 2006; Taylor, 2009; Taylor et al., 1997). The mediators are in part soluble neuropeptides produced by the cells of the ocular microenvironment that make up the blood-ocular barrier, iris, ciliary body, and retina (Cousins et al., 1991; Kaiser et al., 1989; Namba et al., 2002; Taylor et al., 1998). Understanding the mechanisms of immunomodulation within the healthy ocular microenvironment can help find ways to suppress the damaging immunological activity of inflammation associated with autoimmune diseases, unregulated wound repair, and transplantation graft rejection.

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We have recently discovered that several neuropeptides produced by retinal pigment epithelial (RPE) cells suppress endotoxin-induced activity in monocytic leukemic cell lines (Lau and Taylor, 2009; Zamiri et al., 2006). The effects are not just suppression of proinflammatory cytokine production, but also induction of IL-10. Moreover, two suppressors of inflammation within the ocular microenvironment, alpha-melanocyte stimulating factor (α -MSH), and pigment epithelial derived factor (PEDF), are known to induce IL-10 production in endotoxin-stimulated macrophages (Zamiri et al., 2006). This suppression of endotoxin-stimulated activity is not complete. The induction of nitric oxide stimulated by endotoxin is not suppressed, but augmented by α -MSH produced by the RPE (Lau and Taylor, 2009). In addition, RPE induce F4/80⁺ macrophages to be tolerogenic antigen presenting cells (Streilein et al., 2002; Zamiri et al., 2005). These findings have demonstrate that the healthy RPE monolayer promotes immune privilege by regulating macrophage functionality.

Since several immunomodulators produced by RPE stimulate immunosuppressive activity in macrophages, we tested for the possibility that retinal macrophages and microglial cells are manipulated to mediate immunosuppression within the ocular microenvironment. We found that functionality, and survival of primary macrophages was mediated by RPE derived neuropeptides α -MSH and Neuropeptide Y (NPY). Moreover, the retinal microglial cells have characteristics similar to myeloid suppressor cells that is lost in wounded retinas.

2. Materials and Methods

Mice

Female C57BL/6J mice, 6–8 weeks old, were obtained from Jackson Laboratories (Bar Harbor, ME). Pro-opiomelanocortin hormone (POMC) knockout mice, 8 weeks old, were bred in the animal facility of the Schepens Eye Research Institute. Breeding pairs were obtained from Ute Hochgeschwender, Oklahoma Medical Research Foundation. All animal use and care was approved by the Schepens Institutional Animal Care and Use Committee.

Preparation of Posterior Eyecups

Eyecups were prepared as previously described (Lau and Taylor, 2009; Zamiri et al., 2005; Zamiri et al., 2006). Eyes from euthanized healthy mice were enucleated and placed in DMEM (Lonza, Walkersville, PA) on ice for 30 minutes. After excision of the muscles, connective tissue, and conjunctiva, a circumferential incision was performed below the level of the ciliary body, and the remainder of the anterior segment (cornea, iris, ciliary body, and lens) was discarded. The neural retina was gently lifted off the RPE layer by microsurgical forceps. Each neural retina-deficient posterior eyecup, consisting of sclera, choroid, and a monolayer of RPE, were placed into individual wells of a 96-well round-bottom culture plate. The eyecups were submerged in 150 μ L of serum-free media consisting of RPMI 1640, 0.1 M HEPES, NEAA, L- glutamine, sodium pyruvate, 0.1% bovine serum albumin, 1% gentamicin, and supplemented with 0.1 $\times$ ITS+ solution (Sigma Chemical, St. Louis, MO). The conditioned media (CM) from within the eyecup was removed 48 hours after incubation and further diluted 1:4 in serum-free medium. The diluted CM was then used in the assays.

Some of the eyecups were from mice with laser induced retinal damage. The damage was 12 burn spots in each retina using a diode laser. The mice were anesthetized and pupils were dilated using 1.0% tropicamide (Alcon Laboratories, Fort Worth, TX). The retinal burns were made using a diode laser (810 nm; OcuLight SLx; Iris Medical Instruments, Inc., Mountain View, CA) through a slit lamp biomicroscope (Haag-Streit; Mason, OH) and a 22 $\times$ 22 mm coverglass (Baxter Health Care Corp., McGaw Park, IL) as a contact lens. The

burn parameters were chosen to produce a cavitation bubble in the choroid without hemorrhage. This was achieved by limiting the spot size to 100 μm in diameter with the laser energy at 100 mW intensity, and a 100 ms duration of exposure. The 12 laser burns were 4 clusters of 3 burns in the 12, 3, 6, and 9 o'clock positions two optic nerve diameters from the optic nerve head. Three days later, the eyes were collected.

Neutralization of TGF- β , VIP, CGRP, PEDF, α -MSH, NPY and SOM was done by adding to the conditioned media antibody against VIP, CGRP, α -MSH, SOM from Peninsula Laboratories (San Carlos, CA), TGF- β from R&D Systems (Minneapolis, MN), or PEDF from Abcam (Cambridge, MA). For controls, we used ChromPure Rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA). The conditioned media with antibody added was incubated at 37°C for 1 hour, protein-A coated beads (Santa Cruz Biotechnology) were added to the conditioned media and incubated for an additional 1 hour 37°C. The beads were centrifuged down and the supernatant was used in the assays. Antibody concentrations were used at 10 – 100 $\times$ their neutralizing dose.

Immunohistochemistry of retinal section

To stain the retina for Arginase1, NOS2, CD64, CD11b, F4/80, and nitrotyrosine, the freshly-isolated eyes from mice with healthy or laser burned retinas were fixed with 4% paraformaldehyde for 72 hours at 4°C then embedded in paraffin. Vertical 7 mm thick sections of the eye were cut on a cryostat. The sections were warmed for 2 hours at 60°C, deparaffinized in xylene, and dehydrated through a graded series of alcohols. Antigen retrieval for Arginase1, NOS2, CD64, CD11b, F4/80 and nitrotyrosine was done by soaking the sections for 30 min in 90°C water followed by 8 min in 10mM citrate buffer with 0.05% Tween-20 (pH 6), cooled to room temperature and washed in deionized water. Non-specific binding was blocked by incubating the sections in 2 % BSA (Sigma-Aldrich), and depending on the secondary antibodies to be used 100 $\mu\text{g}/\text{ml}$ of mouse or rabbit whole IgG (Jackson ImmunoResearch Laboratory) in 0.01M PBS for 1 hr at room temperature. The sections were stained for two markers as described in the results. The sections were first stained overnight at RT in PBS containing 2 % BSA with the unconjugated antibodies goat anti-arginase1, goat anti-CD64 (Santa Cruz Biotechnology, CA, USA), rat anti-mouse F4/80 (AbD Serotec), or anti-nitrotyrosine monoclonal mouse IgG_{2b,k} (Upstate Lake Placid, NY). The antibodies used for controls were ChromPure mouse IgG, ChromPure rat IgG, ChromPure goat IgG, and ChromPure rabbit IgG (Jackson ImmunoResearch Laboratory). After washing three times with PBS + 0.02% Tween-20, sections were incubated for 4 hours at room temperature in PBS containing 2 % BSA with antibodies to rhodamine-conjugated monoclonal anti-NOS2 IgG (Santa Cruz Biotechnology, CA, USA), PerCP-Cy5.5-conjugated rat anti-mouse CD11b (BD Pharmingen, CA, USA), FITC-conjugated Goat Anti-Rabbit IgG, TIRTC-conjugated donkey Anti-Mouse IgG, or TIRTC-conjugated donkey Anti-Rat IgG (Jackson ImmunoResearch Laboratory). For staining controls at this step we used TRITC-conjugated ChromPure mouse IgG, whole molecule (Jackson ImmunoResearch Laboratory) or PerCP-Cy5.5 conjugated rat IgG_{2b,k} (BD Pharmingen). After washing three times with PBS + 0.02% Tween-20, sections were mounted with mounting medium (Vectashield, Burlingame, CA) and coverslip. Sections were observed under a fluorescent microscopy, and digitally photographed at 200 $\times$ magnification. Presented are representative sections of three consecutive sections selected from one of three different mice treated and handled in the same manner.

Primary macrophages

The primary macrophages were from non-irritated peritoneal cavities. The cells were collected by recovering 8 ml DMEM injected directly into the peritoneal cavity. The cells were centrifuged at 400 $\times$ g for 10 min at 4°C, and suspended in 3 ml DMEM with 5% fetal

bovine serum to assess cell purity and viability. The peritoneal macrophages (1×10^6 cells) were seeded onto glass coverslips placed in the wells of a 24-well flat-bottom culture plate. The cultures were incubated for 90 min at 37°C in 10% CO₂. The plates were washed three times with 1ml/well of RPMI 1640 serum-free medium. These resting macrophage cultures were cultured in 400 µl of 1:4 diluted conditioned media, or in serum-free media containing α-MSH (1 ng/ml) or NPY (1 ng/ml) for 24 h at 37 °C, 10% CO₂ in humidified incubator. The adhered macrophages on the glass cover slips were stained by the same methods and with the same antibodies as the retinal sections for Arginase1, NOS2, and CD11b. The coverslips with the adherent stained cells were mounted to the glass slides with mounting medium, and were digitally photographed under a fluorescent microscope at the same exposure time and intensity at 400 × magnification. Apoptosis was detected in the macrophages by assaying the treated macrophages using a TUNEL assay kit (Millipore-Chemicon, Billerica, MA) and digitally photographed under a fluorescent microscope at 400 × magnification. Presented is the representative result of three separate experiments and staining.

To assay for cytokine production the resting peritoneal macrophages were added at 1×10^6 cells per well of 24-well culture plate in RPMI 1640 with 5% fetal bovine serum for 2 hours, washed twice with RPMI 1640 serum-free medium, and 300 µl of RPMI 1640 serum-free medium added. Into the cultures was added α-MSH and NPY (1ng/ml each), and for a positive control of cytokine production other cultures of macrophages were treated with LPS (1 µg/ml) in 200 µl of serum-free medium. The cultures were incubated for 48 hours at 5% CO₂ at 37°C and the supernatant was assayed using a Bio-Plex Luminex assay (Bio-Rad) to quantify IL-1β, TNF-α, IL-6, IL-10, GM-CSF, and G-CSF in each supernatant. Significant changes in cytokine levels were determined using a Student's t-test with significance at $P \leq 0.05$.

To assay for macrophage mediated apoptosis in T cells, the peritoneal macrophages were added at 1×10^6 cells per well of a 96-well flat-bottom culture plate in RPMI 1640 with 5% fetal bovine serum for 90 mins, washed with RPMI 1640 serum-free medium, and treated with α-MSH and NPY (1ng/ml each) in 200 µl of serum-free medium. The cultures were incubated for 24 hours at 37°C in 10% CO₂. Lymph node cells were collected from the draining lymph node of mice immunized with 50 µg heat killed desiccated mycobacterium (Difco Laboratories, Detroit, MI). A single cell suspension of popliteal lymph nodes (the draining lymph nodes of immunized mice) was made, and the cells were passed through a CD3-enrichment column (R&D Systems). The peritoneal macrophage cultures were washed, and enriched CD3⁺ T cells (4×10^5 cells) were added to the macrophage cultures. The cultures were incubated for 24 hours at 37°C in 10% CO₂. The cells were collected, and subjected to the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore-Chemicon) for flow cytometric analysis. The cells were analyzed using BD LSRII flow cytometer (Schepens Flow Cytometry Facility) gated on lymphocytes, and the percentage of TUNEL positive cells was measured.

Quantification of α-MSH and NPY in the conditioned media

The concentration of α-MSH in the eyecup conditioned media was determined using an ELISA developed in our laboratory (Taylor et al., 1992). The concentration of NPY eyecup conditioned media was determined using an ELISA for NPY from Peninsula Laboratories and used according to the manufacture's specification. Significant changes in neuropeptide levels were determined using a Student's t-test with significance at $P \leq 0.05$.

3. Results

3.1 Ocular derived neuropeptides mediate the co-expression of Arginase1 and NOS2 in macrophages

Previous publications demonstrated that the RPE produces soluble immunomodulatory factors that suppress pro-inflammatory activity in macrophages, and recently we found that RPE may mediate alternative activation of macrophages (Lau and Taylor, 2009; Osusky et al., 1997; Streilein et al., 2002; Zamiri et al., 2005; Zamiri et al., 2006). To assay RPE mediated immunomodulation, we used conditioned media of posterior eyecups with an intact RPE monolayer as published before, and describe in the methods (Lau and Taylor, 2009; Zamiri et al., 2006). This method preserves in culture for a short period of time the in vivo activity of RPE. In addition, the attached choroid and posterior sclera support only conventional activation of macrophages if the RPE layer is removed (Lau and Taylor, 2009). The resting primary macrophages were treated with the conditioned media (CM). The treatment induced co-expression of Arginase1 and NOS2 in the macrophages (Fig. 1). The co-staining reveals the presence of NOS2 near the nuclei of the cells with Arginase1 mainly in the cytoplasm. Also the treated macrophages have smooth membranes and a dendritic-like appearance. This is in contrast to the expression of Arginase1 with little to no expression of NOS2 in untreated macrophages (see Fig. 3). To define the mechanisms of CM induced co-expression of Arginase1 and NOS2 in macrophages, the CM was depleted of specific known constitutively expressed ocular immunomodulating neuropeptides and cytokines (Taylor, 2007). The depletion of only two neuropeptides, alpha-melanocyte stimulating hormone (α -MSH) and neuropeptide Y (NPY), changed the expression of Arginase1 and NOS2 in the CM treated macrophages (Fig. 1). When α -MSH was depleted from the CM, macrophages still expressed Arginase1 and NOS2; however, the pattern of NOS2 staining was no longer nuclear, but more scattered within the cells. In addition, the macrophages changed their appearance, becoming small, rounded and ruffled (Fig. 1). When the CM was depleted of NPY the treated macrophages only expressed Arginase1, and maintained a dendritic-like appearance (Fig. 1). When the CM was depleted of both α -MSH and NPY the treated macrophages expressed only NOS2, and their appearance was similar to the macrophages treated with α -MSH-depleted CM (Fig. 1).

To see if the macrophages that appear small, rounded, and ruffled when treated with α -MSH-depleted CM were undergoing apoptosis, we assayed the treated macrophages by TUNEL (Fig. 1). Almost all of the macrophages treated with CM depleted of α -MSH were TUNEL positive (80% TUNEL positive by flow cytometry), and the same results were seen when the macrophages were treated with CM depleted of both α -MSH and NPY (Fig. 1). There was very little TUNEL staining in the macrophages treated with complete CM, or NPY-depleted CM (Fig. 1). Therefore, both α -MSH and NPY are potentially important factors needed to promote an alternative activation of macrophages with α -MSH an essential factor for macrophage survival in the healthy retina.

Since laser wounded retinas have a disrupted RPE monolayer, a break in the blood-ocular barrier, and have been demonstrated to not support immune privilege and immunosuppression, we assayed the CM of posterior eyecups for α -MSH and NPY by ELISA for immunoregulatory activity. The laser-wounded RPE eyecup CM had significantly lower amounts of α -MSH, and no change in the amounts of NPY when compared to CM of eyecups with healthy RPE (Figs 2A, 2B). The macrophages treated with laser-wounded CM were similar in appearance as the macrophages treated with healthy CM depleted of α -MSH, non-nuclear distribution of NOS2, round appearance and apoptotic (Figs 2C, 2G). Moreover, adding α -MSH into the laser-wounded CM rescued the macrophages from apoptosis, and made the macrophages co-express Arginase1 and NOS2

with NOS2 localized to the nucleus (Figs 2D, 2H). This was similar to what was seen in macrophages treated with healthy RPE CM.

In addition, we assayed the effects of RPE CM of eyecups from mice with the proopiomelanocortin gene knocked out (POMC-ko). The macrophages treated with this conditioned media either expressed Arginase1 or NOS2 along with apoptosis (Figs 2E, 2I). The addition of α -MSH to the POMC-ko CM rescued the macrophages from apoptosis, and the macrophages co-expressed Arginase1 and NOS2 (Figs 2F, 2J). These results further demonstrate an important role for α -MSH in regulating macrophage survivability and functionality in the retina.

3.2 The regulation of macrophages by α -MSH and NPY

The neuropeptides α -MSH and NPY individually and together were assayed for their effects on Arginase1 and NOS2 expression, and cytokine production by macrophages. The untreated resting macrophages predominately express Arginase1 with little NOS2, and were morphologically small, but elongated in culture (Fig. 3). The resting macrophages treated with α -MSH were larger, and somewhat dendritic in shape. These macrophages expressed Arginase1, and relatively little NOS2. The NPY treated resting macrophages were rounder than the α -MSH treated cells, and expressed relatively more NOS2 filling the cytoplasm with little Arginase1 expression (Fig 3). In addition, less of the NPY-treated macrophages adhered to the glass coverslips (Stanojevic et al., 2006) reducing the number of cells that could be stain. The resting macrophages treated with both α -MSH and NPY were larger and some were dendritic in shape. There was co-expression of Arginase1 and NOS2 that was similar to what was seen in macrophages treated with healthy CM (Fig 3). None of the treatments induced apoptosis (Fig. 3). Since resting macrophages were treated, the macrophage cell cultures were assayed to see if the α -MSH/NPY-treatment induced any of the cytokines previously reported (IL-1 β , TNF- α , IL-6, IL-10, GM-CSF, G-CSF) to be affected by eyecup conditioned media treatment (Lau and Taylor, 2009; Zamiri et al., 2006) (Fig. 4). The α -MSH/NPY-treatment did not induce any of the assayed cytokines in the resting macrophages. However, while the production of specific cytokines was not detected, the α -MSH/NPY-treated macrophages did induce apoptosis in T cells that was 3.4-fold over T cells cultured with untreated resting macrophages (data not shown). These results demonstrated that the CM through α -MSH and NPY induces some of the features associated with myeloid derived suppressor cells.

3.3 Analysis of Arginase1 and NOS2 co-expressing cells in the healthy retina

If the α -MSH and NPY produced by the RPE are mediating the co-expression of Arginase1 and NOS2 in macrophages, then such cells should be found in the healthy retina. Retinas of healthy and laser-wounded eyes were co-stained for Arginase1 and NOS2 (Fig 5) and for the macrophage markers CD11b, CD64 and F4/80 (data not shown). In the healthy retina we found that all the cells that stained for macrophage markers also co-staining for Arginase1 and NOS2. These co-staining microglial cells were found mostly at the interfaces of inner plexiform layer with the ganglion cell layer and the inner nuclear layer (Fig 5A). They also populated the outer plexiform layer. We found no microglial cell in the healthy retina that expressed only NOS2 without Arginase1. In contrast the wounded retina had a mixture of single stained cells (Fig 5B) that occupied the same places as in the healthy retina except many were now centered near the wound site. Unlike in the healthy retina there were no microglia co-stained with Arginase1 and NOS2. Instead 89% of the microglia expressed only NOS2, and 11% expressed only Arginase1 (TABLE 1). In both the healthy and wounded retinas there were a population of Arginase1 only stained cells (Fig 5A, 5B); however, they did not stain for any of the macrophage markers.

Since the co-expression of Arginase1 and NOS2 causes production of peroxynitrite (Bronte and Zanovello, 2005) in functional macrophages, if the co-stained cells in the retina are active, then nitro-tyrosine should be seen associated with the co-expressing microglial cells. In healthy retinas there is nitro-tyrosine staining associated with 96% of the CD64⁺ retinal cells (Fig. 5C). In the laser-wounded retina nitro-tyrosine staining was seen with 64% of the CD64⁺ cells, and no nitro-tyrosine staining was associated with 36% of the CD64⁺ cells (Fig. 5D). It is possible that in the wounded retina we are detecting two populations of microglial cells correspond to cells expressing respectively only NOS2 or only Arginase1 as seen in Figure 5C. Therefore, in the healthy retina there are microglial cells co-expressing Arginase1 and NOS2, that are generating peroxynitrite.

4. Discussion

The results demonstrated that the neuropeptides α -MSH and NPY released by the ocular posterior chamber, mostly likely by the RPE, induce co-expression of Arginase1 and NOS2 in resting macrophages that have the potential to act as myeloid suppressor cells. There was co-expressing Arginase1 and NOS2 functional microglial cells in the healthy retina. In contrast, within the wounded retina there was a combination of macrophages and microglial cells expressing either Arginase1, or NOS2, but not both. Like in the anterior chamber of the eye (Taylor, 2007) there is a role for neuropeptides in regulating immunity in the retina. When the integrity of the ocular microenvironment is compromised by a break in the blood-ocular barrier this neuroimmunomodulating activity is also compromised.

Macrophages are a heterogeneous population of immune cells that respond to various endogenous factors that polarized their functionality and associated patterns of cytokines, chemokines, and intracellular enzymes (Gordon, 2007; Mantovani et al., 2004). While the polarization of macrophage functionality can be simply divided into classical and alternative activation; however, there is a wider spectrum of functional macrophages (Gabrilovich and Nagaraj, 2009; Mosser and Edwards, 2008). Somewhere in this spectrum of macrophage activity are macrophages that actively suppress immunity. There is a slow but steady migration of macrophages into the retina (Kaneko et al., 2008; Kezic and McMenamin, 2008; Lehmann et al., 2010; Xu et al., 2007). Also, there is evidence that this maybe a homeostatic replacement and turnover of microglial cells in healthy and injured retinas by monocyte precursors (Kaneko et al., 2008; Xu et al., 2007). The constitutive presence of the neuropeptides means that newly emigrated macrophages and resident microglia will be continuously influenced by the neuropeptides as the cells remain within the retinal tissue microenvironment.

In the healthy retina there was a single CD11b⁺ CD64⁺ F4/80⁺ population of cells, most likely microglial cells, that co-express Arginase1 and NOS2. In the laser-burned retina, these cells can be separated into two different populations where none co-expressed Arginase1 and NOS2. These immunohistochemical results parallel the effects of eyecup-conditioned media on macrophages in culture. The differences seen in the macrophages between the conditioned media of healthy and laser-burned eyecups is related to the presence or absence of α -MSH in the conditioned media. However, the co-expression of Arginase1 and NOS2 required both α -MSH and NPY. Therefore, since we have previously found that alternative activation is dependent on the intact RPE in the eyecup preparations (Lau and Taylor, 2009), the co-expressing microglia in the retina must be related to healthy RPE production of α -MSH and NPY.

The neuropeptide α -MSH is generally considered an anti-inflammatory neuropeptide. It antagonizes endotoxin and IL-1 induced inflammatory activity, and induces anti-inflammatory activity in macrophages (Lipton and Catania, 1997). It also mediates its own

production, and augments expression of its receptors on macrophages establishing a self-sustaining anti-inflammatory autocrine loop (Taherzadeh et al., 1999). In contrast NPY is more pleiotropic in regulating macrophage functionality related to the type of NPY receptor expressed (De la Fuente et al., 2001; Dimitrijević et al., 2008; Dimitrijevic et al., 2005; Zhou et al., 2008). In our results we show that NPY promotes NOS2 expression. This is more of a proinflammatory signal by NPY. What has not been seen before is the effect of α -MSH and NPY together on resting macrophages. The results were cells that co-express Arginase1 and NOS2. The cells appeared to be activated, and while we did not detect the production of a specific set of cytokines, these cells did have a greater capacity to induced apoptosis in T cells. While much more analysis needs to be done, the results suggest cooperation between α -MSH and NPY mediated intracellular-signals to alternatively activate macrophages with potential myeloid suppressor cell functionality.

The cells that make up the posterior of the eye produce soluble factors that clearly regulate macrophage function. Previously we found that RPE is the source of these immunomodulating factors, and that they are a specific set of neuropeptides and growth factors (Lau and Taylor, 2009; Zamiri et al., 2005; Zamiri et al., 2006). The conditioned media alternatively activated the macrophages. Without α -MSH, either through depletion, wounding, or knockout, the conditioned media classically activated the macrophages as seen by changes in macrophage morphology, and expression of NOS2. In addition, the macrophages in these cultures underwent apoptosis. It was possible to use α -MSH to block this apoptotic signal. Blocking of apoptosis with α -MSH has been seen in astrocytes, hypothalamic neurons, melanocytes, and renal tubular cells (Bohm et al., 2004; Caruso et al., 2007; Chai et al., 2006; Lee et al., 2004). However, there is no description of which possible pathway of inducing apoptosis is suppressed by α -MSH, with the exception of a hint that α -MSH increases the ratio between Bcl-2 and Bax in astrocytes altering the intrinsic pathway (Caruso et al., 2007). There are no previous reports that α -MSH modulates apoptosis in macrophages.

The depletion of NPY caused the conditioned media to be even more so an alternative activator of macrophages; however, there was no co-expression of NOS2. Without both α -MSH and NPY the conditioned media induced NOS2 only expressing macrophages with apoptosis. The conditioned media of laser-burned eyes activated the macrophages just like the α -MSH-depleted conditioned media of healthy eyes. This is supported by the diminished expression of α -MSH in the laser-burned conditioned media. Therefore, the RPE in the posterior eyecups produce α -MSH and NPY along with mediators of apoptosis (Griffith et al., 1995; Hueber et al., 2002) and inflammation, which must be antagonized by α -MSH.

Our results demonstrate the RPE as a source of neuropeptides; however, in the retina there are several sources of NPY and α -MSH. The amacrine cells of the inner nuclear and ganglion cell layer are a major source of NPY (Ammar et al., 1998; Bruun et al., 1986; Tornqvist and Ehinger, 1988; Ye et al., 1990). Also, there is the potential for muller cells, and retinal microglial cell to produce NPY (Alvaro et al., 2007). Another retinal source of NPY is from the perivascular space of the central retinal artery associated with peptidergic nerve fibers running parallel with the artery, but stopping at the surface of optic nerve disk (Ye et al., 1990). The sources of α -MSH in the retina are limited. Besides the RPE, α -MSH is found also in the ganglion cell layer (Lee et al., 2009). When the RPE cell layer was disrupted by laser burns the conditioned media could not induce co-expression of Arginase1 and NOS2 in macrophages. There was a similar in vivo change in microglial cell co-expression of Arginase1 and NOS2. The change in the conditioned media was associated with diminished levels of α -MSH. If this is also the case in the laser wounded retina in vivo, it would mean that α -MSH production by ganglion cells is not sufficient to sustain retinal immunoregulation. This further supports the importance of the intact RPE cell layer in

regulating retinal immunity by in part being a major source of α -MSH in the retina (Sugita et al., 2006; Zmijewski et al., 2007).

This study has further demonstrated the dependence of the ocular microenvironment on specific neuropeptides to tightly control the activity of macrophages in maintaining ocular homeostasis. It is conceivable that the healthy retina may select for specific types of monocytes to become resident microglial cells. A monocyte that has migrated into the retina that is not responsive to α -MSH would undergo apoptosis; however, the monocyte that is saved by α -MSH has its fate set by α -MSH, NPY, and other immunomodulating factors like TGF- β 2 to become potential myeloid suppressor cells, or tolerogenic antigen presenting cells. The result would be, as observed, a uniform population of resident suppressor microglial cells in the retina. In addition, potential therapeutic approaches that reintroduce or augment expression of specific neuropeptides may protect the eye from further damage caused by trauma and inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression of Arginase1, NOS2, and TUNEL staining in primary macrophages treated with eyecup conditioned media. Primary macrophages were treated with conditioned media (CM), CM depleted of α -MSH ($-\alpha$ -MSH), CM depleted of NPY ($-\text{NPY}$), or CM depleted of both α -MSH and NPY ($-\alpha$ -MSH $-\text{NPY}$). The treated cells were incubated for 24 hours, and then co-stained with FITC-labeled anti-Arginase1 and PE-anti-NOS2, or assayed for apoptosis by TUNEL staining methods (red stained cells). The results are representative of three separate experiments with three different conditioned media.

Figure 2.

Expression of Arginase1 and NOS2 by macrophages treated with conditioned media of laser-wounded or POMC knock out (POMC(-/-)) eyes. The concentration of (A) α -MSH, and (B) NPY in healthy and laser-wounded CM were assayed by ELISA. Presented are the mean $\pm$ SEM of four eyecup CM from four different mice. *Statistical difference ($P < 0.001$) was determined by Student's T-test. NS= not significantly different. Primary macrophages were treated for 24 hours with (C, G) CM of laser-wounded RPE eyecups, or (D, H) CM of laser-wounded RPE eyecups plus 1 ng/ml of α -MSH added. The treated cells were co-stained with FITC-labeled anti-Arginase1 and PE-anti-NOS2 (C, D), or assayed by TUNEL for apoptosis (G, H). The results are representative of three separate experiments with three different conditioned media. In addition, Primary macrophages were treated with either (E, I) CM of POMC(-/-) eyecups, or (F, J) CM of POMC(-/-) eyecups plus 1 ng/ml of α -MSH added. The treated cells were co-stained with FITC-labeled anti-Arginase1 and PE-anti-NOS2 (E, F), or assayed by TUNEL for apoptosis (I, J). The results are representative of two separate experiments with two different conditioned media.

Figure 3.

The effects of treating macrophages with α -MSH and NPY. Primary macrophages were treated with α -MSH (1ng/ml), NPY (1ng/ml), or both for 24 hours. The cells were co-stained for Arginase1 and NOS2, or assayed by TUNEL staining for apoptosis. All images were made with the same exposure time and subtraction of background. The results are representative of three separate experiments.

Figure 4.

Cytokine pattern of neuropeptide treated macrophages. The primary macrophages were untreated, treated with both α -MSH and NPY, or with LPS. After 48 hours incubation the culture supernatants were assayed for cytokine production using multi-plex analysis. Presented are the results of four independent macrophage samples (mean $\pm$ SD). Significant differences ($\dagger$ $P < 0.05$ or $*$ $P < 0.001$) was determined by Student's T-test analysis in comparison with the LPS-stimulated macrophages.

Figure 5.

The co-expression of Arginase1 and NOS2 in healthy and wounded retinas. Paraffin sections of C57BL/6 mice A) healthy retinas or B) 72 hour laser-wounded retinas were co-stained with FITC-labeled anti-Arginase1 and PE-anti-NOS2. Plain arrows identify double stained cells, and the * arrows mark single stained cells. The expression of nitrotyrosine in C) healthy and D) wounded retinas were done on retinal sections co-stained for nitrotyrosine, and for the macrophage/microglial cell marker CD64. The arrows identify CD65⁺ cells, and * arrows identify CD65⁺ cells that are not associated with nitrotyrosine staining. Inserts are magnified regions boxed in the figure. The results are representative sections selected from three different sections from three different mice.

TABLE 1

Frequency of retinal microglial cells¹ expressing Arginase 1 and NOS2

Healthy Retina ²			Laser Wounded ³		
Arginase1 ⁺ Only	NOS2 ⁺ Only	Arginase1 ⁺ NOS2 ⁺	Arginase1 ⁺ only	NOS2 ⁺ only	Arginase1 ⁺ NOS2 ⁺
0 %	0 %	100 %	11 ± 1 %	89 ± 9 %	0 %

¹ Microglial cells, CD64⁺ F4/80⁺ CD11 b⁺ cells, were counted in retinal sections stained as described in Figure 5 and in the methods.

² All Arginase 1 only expressing cells did not co-express markers of microglia.

³ Non-microglial cells stained for only Arginase 1 (15% of all stained cells)