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Injection of an Alpha-Melanocyte Stimulating Hormone Expression Plasmid is Effective in Suppressing Experimental Autoimmune Uveitis

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Abstract

Purpose—The neuropeptide, alpha-melanocyte stimulating hormone (α -MSH), is an endogenous antagonist of inflammation. Injections of α -MSH peptide into inflamed tissues have been found to be very effective in suppressing autoimmune and endotoxin mediated diseases. We evaluated the potential to suppress ocular autoimmune disease (uveitis) by augmenting the expression of α -MSH through subconjunctival injections of naked adrenocorticotrophic hormone amino acids 1-17 (ACTH1-17) plasmid.

Methods—We clinically scored the uveitis over time in B10.RIII, C57BL/6, and melanocortin 5 receptor knock-out (MC5r^{-/-}) mice with experimental autoimmune uveitis (EAU) that were conjunctively injected with a naked DNA plasmid encoding ACTH1-17 at the time of EAU onset and three days later. The post-EAU retina histology of plasmid injected eyes was examined, and post-EAU concentrations of α -MSH in aqueous humor was assayed by ELISA.

Results—The subconjunctival injection of ACTH1-17 plasmid augmented the concentration of α -MSH in the aqueous humor of all post-EAU mice. The injection of ACTH1-17 suppressed the severity of EAU in the B10.RIII and C57BL/6 mice but the MC5r^{-/-} mice. In all the models of EAU, the ACTH1-17 injection helped to preserve the structural integrity of the retina; however, post-EAU aqueous humor was not immunosuppressive.

Conclusions—The subconjunctival injection of the α -MSH expression vector ACTH1-17 plasmid is effective in suppressing EAU. The suppressive activity is dependent on MC5r expression, and possibly works though α -MSH antagonism of inflammation than on α -MSH directly modulating immune cells. The results suggest that an effective therapy for uveitis could include a gene therapy approach based on delivering α -MSH.

Keywords

Plasmid injection; ACTH1-17; α -MSH; uveitis; experimental autoimmune uveoretinitis

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Introduction

The peptide, alpha-melanocyte stimulating hormone (α -MSH), was originally discovered as a pituitary derived peptide hormone that induced melanogenesis (1). The α -MSH peptide is an endoproteolytic product of the protein, proopiomelanocortin hormone (POMC) gene. POMC is also the pro-protein for adrenocorticotrophic hormone (ACTH), and beta-lipotropin, which are in turn pro-proteins for α -MSH, corticotropin-like intermediate lobe peptide, gamma-LPH, and beta-endorphin (2). POMC is cleaved into a 39 residue peptide by prohormone convertase 1 (PC1). Further cleavage of the 39 residue peptide by PC2 liberates the first 17 residues from which α -MSH is derived. Native α -MSH is formed following cleavage of the C terminal four peptides of the 17 residue ACTH peptide that function as the amidation signal for carboxyl terminal amidation. The native α -MSH peptide has also an acetylated amino terminus.

In mammals, α -MSH suppresses innate immunity and inflammation through several different mechanisms (3). Activation of the central intracellular inflammatory mediator NF- κ B is inhibited by α -MSH (4-6). This inhibition is possibly mediated by α -MSH induction of intracellular IRAK-M that blocks IL-1 and Toll-like receptor intercellular pathways (7). This inhibition of NF- κ B activation by α -MSH results in the suppression of the wide range of inflammatory activity of macrophages and neutrophils (4-6,8). Also, α -MSH inhibits the attraction of macrophages and neutrophils to chemokines (9,10). In addition to anti-inflammatory activity, α -MSH induces IL-10 and promotes its own production and expression of its melanocortin receptors on macrophages and dendritic cells suggesting that it can induce an anti-inflammatory, self-perpetuating, autocrine loop (8,11). Inflammation mediated by adaptive immunity is suppressed by α -MSH inhibition of IFN- γ production by Th1 cells with the possible promotion of regulatory activity (TGF- β production) by Treg cells (12,13). Such immunomodulating activity of α -MSH has promoted the idea that α -MSH is an endogenous antagonist of pro-inflammatory signals and has an important role in immune homeostasis along with its other roles in melanogenesis and metabolism.

Since α -MSH appears to hold an important role in immune regulation it has suggested using α -MSH to treat and suppress endotoxin and autoimmune inflammatory disease. There are publications demonstrating the use of α -MSH peptide injections for the suppression of septic shock, contact hypersensitivity, and allograft survival (14-16). There are also publications demonstrating the possibility of using α -MSH to treat uveitis, inflammation of the eye, by injecting α -MSH peptide systemically into mice with uveitis (13,17,18). It was shown that there is a significant diminishment in the severity of the ocular inflammation in rodent models of endotoxin-induced uveitis, and experimental autoimmune uveitis.

Two gene therapy approaches have been reported using constructs of α -MSH delivered through adeno-associated virus, or as naked plasmid (19-21). In these cases the systemic injection of the gene therapy resulted in significant suppression of mouse experimental autoimmune encephalomyelitis and liver toxicity. One of the naked plasmids actually encodes for adrenocorticotrophic hormone amino acids 1-17 (ACTH1-17) this has an advantage in that its product is the release of natively structured α -MSH peptide and no ACTH (19). This also limits the type of transfected cells that can make α -MSH, since they will need to express the specific enzymes that post-translationally modify ACTH1-17 into native α -MSH. Outside of the neuroendocrine system, these prohormone convertases are found in retinal pigment epithelium, the ciliary body, and macrophages (22-27). Therefore, we examined the potential of injecting the naked plasmid ACTH1-17 to suppress EAU in mice.

Materials and Methods

Animals

Inbred 6-8 week-old BALB/c, B10.RIII, and C57BL/6 mice were used. BALB/c, B10.RIII, and C57BL/6 mice were obtained through Jackson Laboratories (Bar Harbor, ME), and housed in the Schepens Eye Research Institute Animal Facility. All experimental animals were treated in accordance with procedures approved by the Schepens Institutional Animal Care and Use Committee and according to the ARVO statement for the use and care of animals in research.

Induction of Experimental Autoimmune Uveitis

EAU was induced in B10.RIII mice and C57BL/6 mice. To induce EAU in the B10.RIII mice they were subcutaneously injected with 50 μ g of human interphotoreceptor retinoid binding protein peptide spanning amino acid residues 161-180 (IRBPp 161-180) in complete Freund's adjuvant (CFA) fortified with 5 mg/ml heat killed *M. tuberculosis* H37RA (28). To induce EAU in the C57BL/6 mice they were subcutaneously injected with an emulsion of 200 μ g of IRBPp spanning amino acid residues 1-20 (IRBPp 1-20) in complete Freund's adjuvant (CFA) fortified with 5 mg/ml heat killed *M. tuberculosis* H37RA followed by a 0.1 μ g intraperitoneal injection of pertussis toxin (28). The mouse eyes were examined every three to four days by fundus examination, and the severity of retinal inflammation was assessed on a score of 0 - 5 as previously described (12,13). Prior to the examination, the pupils were dilated with topical application of 1.0 % Tropicamide ophthalmic solution (Akorn Inc., Buffalo Grove, IL). Data shown was pooled from 5-9 experiments. In each experiment 5-10 mice per group were used.

ACTH1-17 Plasmid Injections

The α -MSH expression vector was provided to us from Zycos Inc. The expression vector was pCMV-Script with an insert that codes for the region of the POMC gene corresponding to ACTH amino acids 1 - 17 (19). The ACTH1-17 encodes the full length of α -MSH and the four amino acid signaling peptide for alpha-amidation. A closed pCMV-Script plasmid with no insert was used as a control. The treatment was a 5 μ L subconjunctival injection of 1 mg/mL of α -MSH expression vector or empty vector on 6 and 9 days after immunization to induce EAU.

α -MSH Peptide Injections

The α -MSH peptide was purchased from Peninsula Laboratories, Belmont, CA and reconstituted to a concentration of 2 mg/mL in sterile saline. At days 6 and 9 after immunization for EAU, mice received subconjunctival injections of 5 μ L of the α -MSH peptide in sterile saline. Control mice were given 5 μ L subconjunctival injections of sterile saline.

Aqueous Humor Samples

Following resolution of EAU or ten days after injection of the α -MSH plasmid, aqueous humor was collected immediately from both eyes by an anterior chamber puncture (6 μ L/mouse) using a modified glass tube under the surgical microscope. One experiment consisted of aqueous humor samples pooled from five mice and assayed for α -MSH protein concentration. The concentration of α -MSH in the aqueous humor samples was measured by sandwich ELISA (29). The ELISA assays were performed in duplicate or triplicate and the representative data was the mean of aqueous humor samples $\pm$ standard error of the mean (SEM).

T cell Suppression Assay

T cells were enriched from draining lymph nodes 7 days after BALB/C mice were immunized with Complete Freund's adjuvant (CFA) fortified with 10 mg/mL heat killed *M. tuberculosis* H37RA using a CD3 column (R&D systems, Minneapolis, MN). The enriched lymph node T

cells were stimulated with 1 $\mu\text{g/mL}$ of anti-CD3 ϵ antibody (2C11, BD Pharmingen, San Diego, CA), and incubated with pooled aqueous humor of 5 post-EAU mouse eyes. Aqueous humor was diluted 1:4 before adding to the T cell cultures. The cultures were incubated at 37°C, 5% CO₂ for 48 hours, and supernatants were analyzed by ELISA (R&D Systems) for IFN- γ . The experiment was performed in triplicate and results are presented as the mean IFN- γ $\pm$ standard error of the mean (SEM).

Histology and Immunohistochemical Staining

After inflammation subsided, retinas were examined to determine if damage occurred within the retinal layers. The eyes were enucleated and fixed 30 days after immunization to induce EAU in the B10.RIII strain, and 60 days after immunization to induce EAU in the C57BL/6 strains. Eyes were fixed in 10% buffered formalin for 24 hours then embedded in methacrylate and 4-6 μm vertical sections were cut through the papillary-optic nerve axis and stained with hematoxylin and eosin. The morphology core facility of the Schepens Eye Research Institute prepared all histopathological tissue. The specimens were observed at 20 $\times$ magnification using bright field settings. Staining for retinal α -MSH was done using sectioned frozen eyes collected on day 12 after immunization to induce EAU. Unfixed frozen sections were Fc-receptor blocked with sheep serum, rinsed with PBS, and blocked with PBS superbloc (Pierce, Rockford, IL). Blocked sections were then stained with sheep anti- α -MSH polyclonal antibody or with sheep IgG for an isotype control (US Biological), secondary staining was with a FITC-conjugated donkey anti-sheep IgG (Jackson Immunological). The specimens were observed at 20 $\times$ magnification using FITC fluorescence settings.

Statistical Analysis

The results of the quantified α -MSH in aqueous humor are presented as the mean $\pm$ SEM. To assess statistically significant differences, Student's unpaired T test was used, and a P-value less than or equal to 0.05 was considered significant. The EAU score for each mouse is the maximum score of both eyes on a given day. Data shown is the mean of all mice in each group with standard errors. In order to validate the consistency of scoring, another experienced lab member chose a mouse at random to examine and score. EAU scores greater than 1 were in concordance of more than 95%. Statistical significance was determined by the Mann-Whitney nonparametric test with a P-value less than or equal to 0.05 was considered significant.

Results

The effects of injecting the ACTH1-17 plasmid into B10.RIII mice

It has been previously demonstrated that systemic α -MSH gene therapy was effective at diminishing the symptoms associated with EAE (19,20), we evaluated the potential that a similar injection of the ACTH1-17 plasmid would suppress the severity of EAU. We immunized B10.RIII mice for EAU and mice were given an intraperitoneal (IP) injection or a subconjunctival injection of the plasmid. The injections were done at the onset of EAU and two days later, because we previously found that α -MSH treatment was the most effective when the autoimmune disease is active (30). A single injection of the plasmid had an effect on the tempo or severity of EAU (data not shown). There was a significant suppression in the course and severity of EAU when the plasmid was injected into the conjunctiva instead of IP (Fig. 1A, 1B). The IP injection did cause a delay in the onset of EAU, but had no significant influence on severity and tempo of EAU. The results indicate that probably because of its proximal location the subconjunctival injection was effective at the concentration and number of injections to affect EAU.

Since we previously demonstrated that a systemic injection of α -MSH peptide had some effect in suppressing EAU we examined whether a subconjunctival injection of α -MSH peptide was

also as effective as the ACTH1-17 plasmid. In Figure 1C we found that severity of EAU was significantly different between the plasmid injected mice and the peptide injected mice. Also, while there was no significant difference between the empty plasmid injected mice and the α -MSH peptide injected mice there was a trend for more mice to have a milder uveitis. This shows that the α -MSH peptide injection could have some effect, but because it is a bolus of peptide its effects are rapidly diluted, and suggests that the benefit of α -MSH therapy may be from a more sustained presence of α -MSH.

To demonstrate that the ACTH1-17 plasmid augments α -MSH protein expression within the eye we injected healthy B10.RIII mouse conjunctiva with the ACTH1-17 plasmid, twice, 3 days apart, and assayed their aqueous humor and retina for α -MSH protein 10 days later, the length of time for B10.RIII mice reach maximum uveitis after plasmid injection. Aqueous humor from eyes injected with the ACTH1-17 plasmid showed a greater than six fold increase in the amount of α -MSH compared to the aqueous humor from the eyes from mice injected with the empty plasmid (Fig. 2A). The aqueous humor levels of α -MSH in the eyes injected with the empty plasmid were at the expected constitutive levels of α -MSH (29). We stained the retina for α -MSH to see if the injection of the plasmid changed the expression of α -MSH peptide in the retina. Retina of ACTH1-17 plasmid injected eyes showed a marked increase in α -MSH expression in comparison to retinas of eyes injected with the empty vector (Fig. 2B). The increase in α -MSH staining was seen in the RPE, inner limiting membrane and ganglion cell layers. We could not see any of these changes with a single injection of α -MSH plasmid (data not shown). These observations indicated that subconjunctival injections of ACTH1-17 plasmid were sufficient to augment the expression of α -MSH peptide within the retina and in aqueous humor.

The effects of subconjunctival injections of ACTH1-17 plasmid on EAU in B10.RIII mice

Since we found that the subconjunctival injections of the ACTH1-17 plasmid had a significant effect on the clinical severity and the tempo of EAU in the B10.RIII mice we examined the possibility that when EAU is resolved there is also a benefit in preserving the retinal structure. As we did in Figure 1, the B10.RIII mice were immunized for EAU (Day 0), one group received two injections (Day 6 and 9) of the ACTH1-17 naked plasmid, and another group of mice received two injections of empty plasmid. The course of uveitis was followed for both groups. When we compared the mean maximum EAU score for each mouse, there was as shown in Figure 1 a significant reduction in the severity and tempo of EAU in the mice injected subconjunctivally with the ACTH1-17 plasmid (Fig. 3A, 3B). The mean maximum EAU score was 1.9 in α -MSH-plasmid injected mice compared to a mean maximum EAU score of 3.0 in the empty plasmid injected mice.

We examined retinal sections of eyes from mice that had the mean maximum score of the group to see what is the condition of the retina after the ocular inflammation naturally subsided (Fig. 3C). The post-EAU retinas of mice injected with the empty plasmid had the expected disruption of retinal layers, photoreceptor loss, and possible fibrosis or granulomas formations. In contrast, the post-EAU retinas in the ACTH1-17 plasmid injected mouse had normal retinal layers with little to no photoreceptor loss, and some residual infiltrating cells. We also collected the aqueous humor from these post-EAU mice and assayed for α -MSH. The ACTH1-17 plasmid injected group had a significantly higher concentration of α -MSH in their post-EAU aqueous humor compared to the empty plasmid injected group, which had normal levels of α -MSH (Fig. 3D).

The effects of α -MSH plasmid injections on EAU in C57BL/6 mice

The EAU in B10.RIII is an acute and severe form of uveitis. A second model of EAU is in the C57BL/6 mouse where the uveitis is mild, but more persistent (31-33). Therefore, we evaluated

the effects of injecting the ACTH1-17 plasmid into EAU C57BL/6 mice. The severity of the disease was significantly reduced in the mice that were subconjunctively injected with the ACTH1-17 plasmid while duration of EAU was not changed (Fig. 4A, 4B). During the course of EAU, the mean maximum EAU score of the ACTH1-17 plasmid injected group was less than 2 for the entire experiment, and was significantly less than the empty plasmid injected group with a mean maximum EAU score of 3 (Fig. 4B). The histology of the post-EAU retinas show that the retinas of mice injected with the ACTH1-17 plasmid retained their normal retinal structure with some residual infiltrating cells in comparison to the folds and remaining vasculitis in the retinas of post-EAU mice injected with the empty plasmid (Fig. 4C). The post-EAU concentration of α -MSH in the aqueous humor was at least four fold higher in two separate experiments than the aqueous humor concentration in the empty plasmid injected mice (Fig. 4D). Therefore, the injection of the ACTH1-17 naked plasmid is not only effective in increasing the α -MSH protein concentration in the eye, but also effective in suppressing autoimmune uveitis and the associated damage to the retina.

The effect of post EAU aqueous humor on IFN- γ production by activated effector T cells

To understand the immunological mechanism of action by injecting the ACTH1-17 plasmid we examined whether the plasmid injection restored aqueous humor immunosuppressive activity. Aqueous humor is known to suppress the production of IFN- γ by activated effector T cells, and this activity is due to α -MSH in the aqueous humor (34). Since we find that there is an augmentation in the levels of α -MSH in aqueous humor of post-EAU mouse eyes injected with ACTH1-17 plasmid one possible mechanism is that aqueous humor is enhanced in immunosuppressive activity. We activated effector T cells with anti-CD3 antibody and treated the cells with post-EAU aqueous humor and measured secreted IFN- γ with aqueous humor from empty plasmid injected or ACTH1-17 plasmid injected mice. We found that aqueous humor from post-EAU (Day 60) mice injected with empty plasmid cannot suppress T cell production of IFN- γ production, nor did aqueous humor from post-EAU mice injected with ACTH1-17 plasmid (Fig. 5). These results indicate that the injection of ACTH1-17 plasmid does not restore aqueous humor immunosuppressive activity even though the injection elevates α -MSH concentration in the aqueous humor. Moreover, the results suggest that the natural recovery of the mice from EAU is not associated with a recovery of aqueous humor immunosuppressive activity and possible restoration of ocular immune privilege.

The effects of subconjunctival injections of ACTH1-17 plasmid on EAU in MC5r^(-/-) mice

Another possible immunological action is α -MSH directly suppressing T cells activity through the melanocortin 5 receptor (MC5r) on the T cells (12). Without MC5r α -MSH mediated suppression would be indirect with α -MSH binding other melanocortin receptors on other cells that in turn suppress IFN- γ by T cells. We also found that MC5r is highly expressed by cells in the normal retina (35). To see if the MC5r is also required for the suppression of EAU in the ACTH1-17 plasmid injected C57BL/6 mice, we immunized MC5r^(-/-) C57BL/6 mice to induce EAU and treated them with a subconjunctival injection of ACTH1-17 plasmid. In contrast to the effects of ACTH1-17 plasmid injection into wild type mice (Fig. 4) we found no effect of the plasmid injection on the clinical scoring or duration of EAU (Fig. 6A, 6B). However, the retinal histology revealed that the MC5r^(-/-) mice retinas while still extremely damaged received some protection from the ACTH1-17 plasmid injection (Fig. 6C). Despite the clinical and histological observations, the α -MSH concentration in the aqueous humor was greater in two separate experiments by approximately six fold (Fig 6D). The finding that the ACTH1-17 plasmid injection had no effect on the course and clinical score of EAU in the MC5r^(-/-) mice indicates that expression of MC5r is required for the ACTH1-17 plasmid to be effective in suppressing EAU.

Discussion

Over the past decade it has become evident that the healthy ocular microenvironment engages the immune system to prevent the induction of inflammation and possibly manipulate immunity to regulate itself (30,36-42). The mechanisms of immune privilege include the constitutive presence of specific immunomodulating neuropeptides (43). One of these neuropeptides is α -MSH (29,44). In aqueous humor α -MSH prevents the activation of effector T cells, suppresses the inflammatory activity of macrophages, and promotes immune cells to produce anti-inflammatory cytokines including more α -MSH. Therefore, part of any goal to reestablish or reimpose immune privilege would have to include introducing or augmenting the concentration of α -MSH within the ocular microenvironment to counter proinflammatory activities within the uveitic eye. Our goal in this manuscript was to demonstrate that an injection of an α -MSH expressing plasmid, as a type of gene therapy, could at least impart some benefit in reducing the severity of EAU. Our results show that the injections of ACTH1-17 naked plasmid were effective in suppressing the severity and duration of autoimmune uveitis, and in reducing the uveitis associated retinal damage.

There are only a limited number of publications on the potential of an α -MSH gene therapy approach to treat autoimmune disease (19,20). Both of these publications used an α -MSH expression plasmid to treat the mouse model of experimental autoimmune encephalomyelitis (EAE), but used different methods of plasmid delivery. In one method the α -MSH gene was packaged into AAV, and then used to transfect autoantigen specific EAE T cells (20). This method had the very T cells that migrate into the CNS produce α -MSH to antagonize the proinflammatory activity in the target tissue. The second method, the source of the plasmid we used in our experiments, injected an ACTH1-17 naked plasmid intramuscularly in the EAE mice (19). This was to see if a sustained systemic expression of α -MSH had an effect on EAE. In both manuscripts the systemic injections did diminish EAE; however, the transfected T cells were more effective, probably due to the T cells delivering α -MSH at the sites of autoimmune T cell activation. Since the naked ACTH1-17 plasmid experiments were injected into the animal instead of transfecting specific cell lines, and that it was effective in suppressing EAE, we used this non-viral delivery system to see if this would affect EAU, and it does. Additionally, the use of naked plasmid gave us the flexibility to examine two different types of EAU mouse models. EAU in C57BL/6 mice is typically a less severe disease and lasts 60 days or more compared to the more severe, short course of 30 days for EAU in the B10RIII mice. In both EAU mouse models the two conjunctival injections of the ACTH1-17 plasmid reduced the severity of the EAU and the associated damage to the retina.

The use of α -MSH as an immunosuppressive therapy is not a new idea. There are publications suggesting the use of α -MSH peptide injections for the suppression of septic shock, contact hypersensitivity, and allograft survival (14-16). We preliminarily demonstrated the possibility of using α -MSH to treat uveitis, by injecting α -MSH peptide systemically, in mice at the peak of EAU (45). The systemic injection of α -MSH peptide accelerated the resolution of EAU in the mice. Others have also used a systemic injection of α -MSH peptide to suppress endotoxin induced uveitis in rats (17,18). They showed a similar diminishment of inflammation within the uveitic eye. These studies looked at the acute effect of the α -MSH peptide injection, and it is uncertain whether the treatment mechanisms were through a systemic suppression of inflammation or actually had a direct effect within the ocular microenvironment. Our subconjunctival injections of ACTH1-17 plasmid augment the concentration of α -MSH within the ocular microenvironment, and seems to have a lasting effect on α -MSH concentration in aqueous humor. Of interest would be whether a similar effect can be induced if the plasmid were injected before induction of uveitis as a preventative treatment, or in the case of the C57BL/6 mouse EAU during the chronic phase to see if it ameliorates the disease after some

damage has occurred. Such questions would be answered while developing an effective α -MSH based therapy.

The subconjunctival injections of the plasmid could go systemic and into regional lymph nodes. While it is probable that the plasmid has made it into the regional lymph nodes, the lack of an effect on EAU by an IP injection of the same amount of plasmid suggests that if it is going into the blood and going systemically that it is probably being diluted to an ineffective concentration or naturally cleared. This does not exclude the possibility that there is an effective systemic concentration of the plasmid that could suppress EAU. While there is a possibility that the conjunctival injection of the ACTH1-17 plasmid has gone systemic or into the regional lymph nodes, the injections have a significant effect on the severity and tempo of EAU, and on the expression of α -MSH peptide within the ocular microenvironment.

It is not certain what is the exact mechanism that elevated α -MSH protein concentration in the eyes when subconjunctively injected with the ACTH1-17 plasmid. There are several possibilities, and each mechanism will need to be evaluated. The first possible mechanism is that the plasmid can diffuse into the eye. However, this is unlikely to be the only cause because of the short half life of naked DNA. A second possible mechanism is that the plasmid transfects cells within the conjunctiva and tissues surrounding the eye, and it is the α -MSH produced outside the eye that diffuses inward. Because of the small size of α -MSH, 1.6 kDa, it should readily diffuse into the eye. An elevated presence of α -MSH could then trigger retinal cells like microglia to enhance their own production of α -MSH. A third possibility is that since macrophages produce α -MSH in an autocrine manner, they could be what is transfected as they are migrating into the eye, with greater migration in the uveitic eye, delivering the α -MSH peptide into the ocular microenvironment and triggering other cells to produce α -MSH. Despite whether one or more of these mechanisms could be active, the conjunctival injections of the ACTH1-17 plasmid increases the intraocular α -MSH concentrations and was effective in diminishing uveitis in EAU.

The classic aqueous humor suppression assay demonstrates the suppressive ability of aqueous humor (34) and it has been previously shown that α -MSH has suppressive effects on innate immunity and adaptive immunity (4-6,8-10,13-15). We found the immunosuppressive neuropeptide, α -MSH, to be abundant in aqueous humor after EAU resolves; therefore, would expect the post-EAU aqueous humor to be immunosuppressive. However, we did not observe a significant suppression in IFN- γ production by activated effector T cells treated with aqueous humor from plasmid injected or empty plasmid injected mice. The inability of post-EAU ACTH1-17 plasmid injected aqueous humor to suppress IFN- γ production by stimulated T cells suggests that this therapy does not act by restoring the immunosuppressive local ocular microenvironment. The finding that the ACTH1-17 plasmid injection had no effect on the course and clinical score of EAU in the MC5r^(-/-) mice should have been an indicator that elevated concentration of α -MSH in the ocular microenvironment is working by suppressing T cell activation; however, because the post-EAU aqueous humor, even with elevated α -MSH levels, cannot suppress effector T cell activation. This means that the suppression of EAU seen by injecting ACTH1-17 plasmid is α -MSH acting through MC5r and antagonizing inflammatory mediators (3), and not necessarily through direct suppression of T cell activation. Moreover, since the MC5r^(-/-) mice recover from EAU like the wild type mice, it suggests that α -MSH mediated immunosuppression is not needed for the natural recovery from EAU. Therefore, the ACTH1-17 plasmid injection must not be reestablishing immunosuppression of immune privilege within the ocular microenvironment, but is elevating the levels of α -MSH in the microenvironment that antagonize pro-inflammatory mediators, possibly providing a neurotropic benefit to the retina, and thus diminish the severity of EAU. Such an antagonistic suppression of inflammation is the immunological action of α -MSH used to treat endotoxin induced uveitis (17,18).

Our results demonstrate the effectiveness of subconjunctival injections of an α -MSH encoded plasmid to diminish the severity, the duration, and the damage of autoimmune uveoretinitis. It also promotes the possibility that there may be an effective therapeutic approach to treating autoimmune uveitis or other autoimmune diseases that is α -MSH based.

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Figure 1.

Effect of subconjunctival and intraperitoneal (IP) injection of naked ACTH1-17 plasmid on uveoretinitis. Mice were immunized to induce EAU and on 6 and 9 days after immunization ACTH1-17 plasmid or empty plasmid was injected. A) The mean EAU scores of each treated group of mice over time. B) The maximum EAU scores of each group. The horizontal line indicates the mean score for each group. The maximum clinical EAU scores of the subconjunctival ACTH1-17 plasmid injected group of mice are significantly ($P < .05$) lower than the maximum clinical EAU scores of the other plasmid injected groups of mice. C) The maximum EAU scores of mice that were injected with empty plasmid, ACTH1-17 plasmid, or with α -MSH peptide. The mean of each group is indicated by the horizontal line. The maximum EAU clinical scores of mice injected with the ACTH1-17 plasmid were significantly lower ($P < .05$) than the maximum EAU clinical scores of the other treated groups of mice.

Figure 2.

Expression of α -MSH within the ocular microenvironment of B10.RIII mice after subconjunctival injection of naked ACTH1-17 plasmid. A) Aqueous humor was collected 10 days after the second injection with empty plasmid or ACTH1-17 plasmid, and assayed for α -MSH by ELISA. The results are the mean $\pm$ standard error of the mean of four different pooled aqueous humor samples. The aqueous humor concentration of α -MSH in eyes injected with ACTH1-17 plasmid was significantly ($P < 0.05$) higher than the aqueous humor concentration of α -MSH from empty plasmid injected eyes. The concentration of α -MSH in the aqueous humor of empty plasmid injected eyes is at the expected ocular physiological concentration of α -MSH. B) The retinas of mouse eyes 10 days after subconjunctival injection with either

ACTH1-17 plasmid or empty plasmids were immunostained for α -MSH peptide. In the empty plasmid injected eyes, α -MSH expression can be seen in the RPE, inner limiting membrane, and ganglion cell layers. Some autofluorescence is observed in the isotype controls in the RPE, outer plexiform layers, and in the choroid. However, greater levels of α -MSH expression were seen in the RPE, inner limiting membrane, and ganglion cell layers of the ACTH1-17 plasmid injected mice.

Figure 3.

The effects of a subconjunctival injection of ACTH1-17 plasmid on the B10.RIII mouse model of EAU. Mice were immunized to induce EAU, and on 6 and 9 days after immunization ACTH1-17 plasmid or empty plasmid was injected into the subconjunctiva as we did in Figure 1. A) The mean EAU scores of each group of treated mice over time. There is significant ($P < 0.05$) difference between the EAU scores from days 9 to 30. B) The maximum clinical EAU scores of uveitis for each mouse in each group are presented. The mean maximum clinical EAU score of each group is indicated with a horizontal line. The two groups are significantly different ($P < 0.0001$). C) Histology of sections of the posterior segment of post-EAU B10.RIII mice were taken that were either subconjunctively injected with ACTH1-17 plasmid or the empty plasmid on days 6 and 9 after immunization to induce EAU. The sections presented were from mice that in the course of EAU had a maximum uveitis score that was equal to the mean of their respective groups. The EAU eyes injected with the empty plasmid had as expected areas of disorganized retinal layers, folding(*) and possible granulomas formations(g). In comparison, the eyes of mice injected with the ACTH1-17 plasmid maintained organized and intact retinal layers with some evidence of inflammation. D) After resolution of EAU (Day 30) the aqueous humor was collected from the mice and assayed for α -MSH by ELISA. Data shown is from three separate experiments and are the mean and standard error of the mean (SEM) for each group. One experiment consisted of aqueous humor samples pooled from five mice.

Figure 4.

The effects of a subconjunctival injection of ACTH1-17 plasmid on the C57BL/6 mouse model of EAU. Mice were immunized to induce EAU, and on 6 and 9 days after immunization ACTH1-17 plasmid or empty plasmid was subconjunctively injected as we did in Figure 3 to the B10.RIII mice. A) The mean clinical EAU scores of each group of treated mice over time. There is significant difference ($P < 0.05$) in the EAU scores between the groups from day 15 through day 50. B) The maximum clinical EAU score for each mouse in each group is presented. The mean of each group is indicated with a horizontal line within each group. The two groups are significantly different ($P < 0.0001$). C) Histology of sections of the retina of post-EAU C57BL/6 mice subconjunctively injected with ACTH1-17 plasmid or empty plasmid on days 6 and 9 after immunization to induce EAU. The sections presented were from mice that in the course of EAU had a maximum uveitis score that was equal to the mean of their respective groups. As in the B10.RIII EAU model, EAU eyes injected with the empty plasmid had areas of disorganized retinal layers(*) with vasculitis(v). In comparison, the eyes of mice injected with the ACTH1-17 plasmid maintained organized and intact retinal layers with evidence of minor cellular infiltrates. D) After the resolution of EAU (Day 60) the aqueous humor was collected from the mice and assayed for α -MSH by ELISA. Data shown are the values obtained from two separate experiments for each group. One experiment consisted of aqueous humor samples pooled from five mice.

Figure 5.

Effect of post-EAU aqueous humor IFN- γ production by activated effector T cells. Primed T cells were collected from the draining lymph nodes of immunized mice, stimulated with 2C11, and treated with post-EAU aqueous humor from mice treated with injections of ACTH1-17 plasmid, or empty plasmid. Aqueous humor was pooled from five B10.RIII mice, each group was done in triplicate. There is no statistical difference in the concentration of IFN- γ between cultures of activated T cells treated with aqueous humor from the empty plasmid injected mice and the ACTH1-17 plasmid injected mice.

Figure 6.

The effects of a subconjunctival injection of ACTH1-17 plasmid on MC5r^(-/-) C57BL/6 mouse model of EAU. Mice were immunized to induce EAU, and on 6 and 9 days after immunization ACTH1-17 plasmid or empty plasmid was injected as we did in Figure 4 to the wild type C57BL/6 mice. A) The mean EAU scores of each group of treated mice over time. There are no significant differences in the mean EAU scores between the groups throughout the course of EAU. B) The maximum clinical EAU score for each mouse in each group is presented. The mean of each group is indicated with a horizontal line within each group. The two groups are not significantly different. C) Histology of sections of the posterior segment were taken 60 days after immunization to induce EAU from mice with their eyes injected with α -MSH plasmid or empty plasmid as in A. The EAU eyes injected with the empty plasmid had areas of disorganization and lost retinal layers especially the loss of photoreceptors. In comparison, the eyes of mice injected with the ACTH1-17 plasmid while severely damaged still have clearly discernable layers of the retina. D) After the resolution of EAU (Day 60) the aqueous humor was collected from the mice and assayed for α -MSH by ELISA. Data shown are the values obtained from two separate experiments for each group.