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SVα-MSH, a novel α-melanocyte stimulating hormone analog, ameliorates autoimmune encephalomyelitis through inhibiting autoreactive CD4$^+$ T cells activation

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ABSTRACT

Alpha-melanocyte stimulating hormone (α-MSH) plays a crucial role in the regulation of immune and inflammatory reactions. Here we report that SVα-MSH, a novel α-MSH analog, could ameliorate the clinical severity of experimental autoimmune encephalomyelitis (EAE) in a preventive and therapeutic manner. SVα-MSH treatment induced the production of regulatory T (Treg) cells and reduced the Th17 cells in the CNS of EAE mice. SVα-MSH-treated PLP peptide 139–151-specific T cells showed a down-regulation of T cell activation markers CD69 and CD134. SVα-MSH did not induce apoptosis but blocked the G1/S phase transition, reduced the expression of cyclin E, Cdk2 and the activity of NFAT and AP-1 transcription factors. Thus, SVα-MSH acts as a novel immunotherapeutic approach in the treatment of autoimmune attack on the CNS.

1. Introduction

The tridecapeptide, α-melanocyte stimulating hormone (α-MSH) is derived from the proopiomelanocortin by post-translational processing (Luger and Brzoska, 2007). α-MSH is an endogenous peptide without evident toxicity and has been demonstrated to be safe when given in large and continuous doses to animals and humans (Luger and Brzoska, 2007; Catania, 2008). Recent studies revealed that α-MSH promotes the restoration of injured nerves and the spinal cord (Catania and Lipton, 1994; Joosten et al., 1999; Bharne et al., 2011; Turkoglu et al., 2012). These immunoregulatory influences and nerve restoration suggest that α-MSH could also have beneficial effects in other central nervous system (CNS) autoimmune disorders. In fact, we and others have reported that α-MSH could prevent and treat experimental autoimmune encephalomyelitis (EAE), a disease model for multiple sclerosis (MS) (Yin et al., 2003; Han et al., 2007; Brod and Hood, 2008; Taylor and Kitaichi, 2008). However, α-MSH is very unstable *in vivo* and it lacks selectivity for melanocortin receptors (MC1R to MC5R) (D'Agostino and Diano, 2010). A novel α-MSH analog was designed by replacing Tyr, Ser and Phe with Ser, Ile and D-Phe at positions 2, 3 and 7, respectively. The resulting Ac-SV-α-MSH analog was found to be a selective agonist for MC1R and MC5R (Ying et al., 2007). However, the biological potency and the molecular mechanisms underlying the immunomodulatory activities of SV-α-MSH were not yet completely understood.

EAE is a CD4$^+$ T cell-mediated disease characterized by inflammation and demyelination within the CNS (Zamvil and Steinman, 1990). In the SJL/J mouse, relapsing/remitting EAE (REAE) can be induced by active immunization with the immunodominant epitope of proteolipid protein $PLP_{139–151}$ (Brown and McFarlin, 1981). The disease is characterized by chronic or relapsing/remitting paralysis due to autoreactive CD4$^+$ Th1 cells that infiltrate the brain and spinal cord and damage the self antigen myelin (McRae et al., 1992). The activation of CD4$^+$ autoreactive T cells and their differentiation into a Th1 phenotype are crucial events in the initial steps, and these cells are probably also important players in the long-term evolution of the disease.

In the present study, we examined the effects of SVα-MSH on clinical outcome in the EAE model *in vivo*, a prototypic model of organ-specific autoimmunity. We demonstrate that the signs of REAE in SJL/J mice can be ameliorated by SVα-MSH *in vivo* both in a preventive and therapeutic fashion. CNS inflammation associated with EAE was significantly reduced. Our data indicate that this therapeutic effect is caused by a down-regulation of Th1 and Th17 immune response, as confirmed by reduced CD4$^+$ Th17 cells infiltrate and increased number of CD4$^+$CD25$^+$ Foxp3$^+$ Treg cells in the CNS. Because T cells also have melanocortin receptors for α-MSH (Andersen et al., 2001; Andersen et al., 2005), we examined effects of SVα-MSH on the activation of

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antigen-specific T cell *in vitro*, such as proliferation, cytokine secretion, cell activation marker expression, cell cycle progression and the activity of nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) transcription factors. Thus, our findings could have relevance for the use of SVα-MSH as a novel immunotherapeutic agent in the treatment of MS.

2. Materials and methods

2.1. Peptides and mice

SVα-MSH (Ac-Ser-Ser-Ile-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) was synthesized by G L Biochem (Shanghai, China) Ltd. The purity of the peptide was greater than 95% as measured by amino acid analysis by HPLC. α-MSH was purchased from Sigma-Aldrich (St. Louis, MO). The CNS target peptide, $PLP_{139–151}$ (HSLGKWLGHPDKF), was obtained from New England Peptide (Fitchburg, MA, USA). Six-week-old SJL/J female mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in groups and maintained on a 12 h light/dark cycle with food and water available *ad libitum*. All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. EAE induction and SVα-MSH or α-MSH treatment

REAE was induced as described previously (Han et al., 2007). Briefly, mice were immunized subcutaneously with 200 μg of $PLP_{139–151}$, emulsified in CFA (Difco, Detroit, MI, USA) plus 250 ng of pertussis toxin (Sigma, St. Louis, MO, USA) in PBS intraperitoneally on days 0 and 2. After 7 days, mice received an identical boost immunization with PLP139–151/CFA, but without pertussis toxin.

Mice were treated with SVα-MSH (0.5 mg/kg) or α-MSH (2.5 mg/kg) once a day by intravenous (i.v.) injection. Coded samples of SVα-MSH or α-MSH dissolved in phosphate-buffered saline (PBS) or vehicle (PBS alone) were administered i.v. by a non-informed investigator. The treatment was started on the same day as $PLP_{139–151}$ peptide was used for the induction of REAE and continued for 5 consecutive days (from day 0 to day 4). The second approach was that the treatment was started right after the onset of the induced disease and continued for 5 consecutive days (day 13 to day 17).

Mice were examined daily for clinical signs of the disease for up to 40 days after the induction and treatment. Mice were scored daily in a blinded fashion using standard criteria as follows: 0, no detectable sign of EAE; 1, loss of tail tone; 2, hind-limb weakness; 3, hind-limb paralysis; 4, hind-limb and forelimb paralysis; 5, moribund or dead.

2.3. Sample preparation and histology

Mice were sacrificed on day 18 after immunization by CO_2 asphyxiation and immediately perfused *via* the left ventricle with sterile PBS. The brain and the spinal column of each mouse were removed and snap frozen in liquid nitrogen and kept at −80 °C until used. For histopathological study, tissues were fixed in 10% buffered formalin. The brain and the spinal cord were dissected and paraffin embedded prior to staining with H&E to assess inflammatory cell infiltration. Mononuclear cells were isolated by digestion of the brain and spinal cord homogenate with collagenase II (1 mg/ml; Sigma, 37 °C, 20 min) and DNAse, followed by discontinuous Percoll gradient (Pharmacia, Piscataway, NJ) Percoll gradient centrifugation. Mononuclear cells at the interface between the 2 gradients (37% and 70% Percoll) were collected and washed by centrifugation with medium.

2.4. Generation of $PLP_{139–151}$-specific T cells

$PLP_{139–151}$-specific T cells (T_{PLP} cells) were generated as described before (Han et al., 2007). $CD4^+$ T cells were cultured with 50 IU/ml recombinant mouse IL-2 (BD, PharMingen, San Diego, CA), harvested after six days, reactivated with $PLP_{139–151}$ peptide plus feeders in 24-well plates (5×10^5 cells/well) and expanded conventionally by alternate activation/rest cycles with antigen/IL-2. T_{PLP} cells were used for experiments starting after the third round of antigen-specific stimulation, when proliferation only occurred in response to the specific antigen, as measured by the incorporation of [^{3}H]-thymidine.

2.5. Proliferation assays and cytokine analysis

T_{PLP} cells (1.0×10^4/well) were co-cultured with irradiated SJL/J splenocytes (1.0×10^5/well) and stimulated with $PLP_{139–151}$ (25 μg/ml) or with plate-bound anti-CD3ε mAb (145.2C11; BD PharMingen, San Diego, CA.) and soluble anti-CD28 mAb (37.51; BD PharMingen) or the vehicle in the absence or presence of different concentration of SVα-MSH (10^{-8} M to 10^{-14} M) as indicated. Lymph node-derived T cells (LNC) were obtained from REAE mice, treated with PBS or SVα-MSH from day 0 to day 4 or on days 13–17, 18 days after immunization with $PLP_{139–151}$ and stimulation with $PLP_{139–151}$ (0.25 μg, 2.5 μg or 25 μg). After 48–60 h' culture, cell supernatants (100 μl) were removed from individual wells and frozen at −80 °C for cytokine assay. Quantitative ELISA assays for IL-17A, IL-4, IFN-γ and TGF-β were performed by using paired mAbs specific for the corresponding cytokines (BD PharMingen), according to manufacturer's recommendations. Proliferation assays were performed in flat-bottomed 96-well microtiter plates. The remaining cells were incubated for an additional 16 h, pulsed with 1 μCi/well of [^{3}H] thymidine (Amersham Pharmacia Biotech), harvested on glass-fiber filters using a Tomtec (Orange) 96-well cell harvester, and radioactivity was counted. Results were expressed as the mean cpm ± SD for triplicate cultures.

2.6. Flow cytometry assays

Monoclonal Abs: FITC-anti-CD4 (RM4-5), APC-anti-CD25 (7D4), APC-anti-IFN-γ-PE (XMG1.2), PE-anti-IL-4 (11B11), APC-anti-CD69 (H1.2F3), PE-anti-CD134 (OX86), and purified anti-FcR were purchased from BD PharMingen (San Diego, CA). PE-anti-CD69 (H1.2F3), PE-anti-CD134 (OX86), PE-Cy7 anti-IL-17A mAb (TC11-18H10.1) and PE anti-Foxp3 mAb (150D) were purchased from BioLegend (San Diego, CA).

For intracellular cytokine staining, isolated CNS-infiltrating cells were stimulated with lymphocyte activator mixture (PMA/ionomycin/brefeldin A, BD Pharmingen) for 5 h and labeled with surface markers FITC anti-CD4 mAb and APC anti-CD25 mAb. After washing, fixing and permeabilizing according to the manufacturer's instructions (BD PharMingen), cells were labeled intracellularly with APC anti-IFN-γ mAb, PE anti-IL-4 mAb, PE-Cy7 anti-IL-17A mAb, or PE anti-Foxp3 mAb or an isotype-matched PE-conjugated rat IgG1 (30 min, on ice). The cells were then washed in 1% FCS/PBS, resuspended in 1% formaldehyde, and analyzed by flow cytometry.

For apoptosis or the cell cycle analysis, T_{PLP} cells (1.0×10^4/well) were stimulated with $PLP_{139–151}$ (25 μg/ml) or the vehicle in the absence or presence of different concentration of SVα-MSH (10^{-8} M to 10^{-14} M) in RPMI 1640 with 10% fetal calf serum (FCS) for 48 h at 37 °C in 5% CO_2 in 24-well plates. Cells were washed twice with PBS, a total of 10^5 T_{PLP} cells/tube were resuspended in a FACS buffer and stained with PE-anti-CD69, PE-anti-OX40 and purified anti-FcR mAbs for 30 min on ice. Flow cytometry was performed by a FACScan flow cytometer with CellQuest software (BD Biosciences, Mountain View, CA). Dead cells were excluded by propidium iodide (PI) (Sigma). To analyze cell cycle progression, cells were collected, washed twice with PBS and resuspended in 300 μl of PBS. 700 μl of ethanol 70% were added slowly to the cells on a vortex and kept at −20 °C for 1 h. PI