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# ALPHA-MELANOCYTE STIMULATING HORMONE SUPPRESSES THE PROLIFERATION OF HUMAN TENON'S CAPSULE FIBROBLAST PROLIFERATION INDUCED BY TRANSFORMING GROWTH FACTOR BETA 1

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**Alpha-melanocyte stimulating hormone (α-MSH) is a proopiomelanocortin derivative and a multi-function neuropeptide, well know for its pigment-inducing capacity, inhibitory action on proinflammatory cytokines and chemoattractant cytokines, and suppressive action on collagen synthesis. Human Tenon's capsule fibroblasts (HTF) are the main effector cells in the initiation and mediation of wound healing and fibrotic scar formation after trabeculectomy. In this study the effects of α-MSH on proliferation of HTF stimulated by transforming growth factor β1 (TGF-β1), have been investigated and discussed. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) in the control group, and in DMEM with TGF-β1 at concentration of $10^{-12}$ M in the TGF-β1 group, and DMEM with $10^{-12}$ M TGF-β1 and α-MSH ranging from 0, $10^{-8}$ to $10^{-4}$ M in the TGF-β1/α-MSH groups. Cell proliferation was assessed 48 h later by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. After administration of TGF-β1 at a concentration of $10^{-12}$ M, or TGF-β1 at $10^{-12}$ M plus α-MSH at $10^{-6}$ M, the mRNA level of type I (α1) collagen, fibronectin, TNF-α, intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), MMP-1, MMP-2, TIMP-1, and TIMP-2 in HTF were analyzed using the real time reverse transcription polymerase chain reaction. α-MSH demonstrated an inhibitory effect on the proliferation of HTF induced by TGF-β1 in a dose-dependent manner, when the concentration was lower than $10^{-5}$ M, and a suppressive effect on the mRNA expression of type I (α1) collagen, TNF-α, ICAM-1 and VCAM-1, which were up-regulated by TGF-β1. Our results showed a reverse effect of α-MSH on the imbalance between MMPs and TIMPs compared with TGF-β1. Based on all these results, we conclude that α-MSH could effectively suppress HTF proliferation and modulate correlative genes in collagen synthesis stimulated by TGF-β1, which implies that α-MSH could be exploited in the treatment of conjunctival fibrotic scar disorder.**

*Keywords*: **alpha-melanocyte stimulating hormone, human Tenon's capsule fibroblast, transforming growth factor beta 1.**

## INTRODUCTION

Trabeculectomy is the most frequently used surgical method for reducing intraocular pressure in patients with unresponsive to medical therapy glaucoma. However, the excessive postoperative healing response can make the desired outcome of such surgery unachievable, when scar formation increases the outflow resistance of the artificially created drainage pathway [1, 2]. Introduction of anti-scarring agents, mitomycin-C and 5-fluorouracil has improved the results of filtration surgery, and the antifibrotic effect of those antimetabolites has been shown to derive mostly from the inhibition of human Tenon's capsule fibroblast (HTF) proliferation [3], as well as from apoptotic cell death [4, 5]. However, these therapeutic applications are associated with severe and potentially blinding complications [6–8], such as bleb leaks that might lead to endophthalmitis, and chronic hypotony. Alternative and safer agents are therefore necessary, especially those with more physiological actions.

After injury, quiescent fibroblasts in the surrounding matrix are activated. They proliferate, and migrate into the wound site to deposit and remodel a new matrix, resulting in tissue fibrosis and scar formation. This process involves a change in the expression of the extracelluar matrix (ECM) proteases (matrix metalloproteinases, MMPs) or their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), and an increase in the synthesis of type III ($\alpha_1$) collagen (collagen involved in the early phases of wound healing), type I ($\alpha_1$) collagen (collagen involved in the late phases of wound healing), and fibronectin (noncollagenous protein) [9, 10]. The process is driven by signaling pathways mediated by proinflammatory cytokines such as the transforming growth factor (TGF-β1) [11, 12].

α-Melanocyte stimulating hormone (α-MSH) is a 13-amino acid neuroimmunomodulatory peptide that arises from the proteolytic processing of the proopi-

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omelanocortin precursor molecule (POMC). It plays a role in the control of melanogenesis and endocrine metabolism, and has a strong anti-inflammatory effect [13–15]. A previous study revealed that α-MSH reduced endotoxin-induced uveitis [16, 17]. We have also demonstrated the anti-inflammatory effect of α-MSH in animal eyes undergoing intraocular surgery [18]. A recent report showed that collagen metabolism was a novel target of α-MSH [19]. It attenuated procollagen synthesis in experimental heart transplantation and reduced tubulointerstitial fibrosis in cyclosporine A (CsA)-induced nephrotoxicity [20, 21]. Besides, α-MSH was known to increase collagenolytic activity by activating matrix metalloproteinases [22, 23]. Hence, the α-MSH peptide may be a potential agent for therapeutic use in treating scar formation after filtering surgery. However, its effect on the wound-healing activities of HTF is unknown. Therefore, the aim of the presented study was to elucidate the effect of α-MSH on HTF proliferation and correlative molecules in collagen synthesis to determine its suitability as an anti-scarring agent for *in vivo* application after glaucoma filtration surgery.

## EXPERIMENTAL

**Cell culture.** Tissue explants of human Tenon's capsule were obtained from three male patients (aged 42, 52, and 59 without any topical eye treatment), who had undergone cataract surgery. Patients were informed of the nature and possible consequences of the tissue removal procedure, and a written informed consent was obtained. The tenets of the Declaration of Helsinki were followed, and approval by the Human Experimentation committee of the affiliated 2nd hospital, College of Medicine, Zhejiang University was granted. Human Tenon's fibroblasts were propagated from 0.5 $cm^3$ tissue explants as previously described [24]. Fibroblasts used in the study were cultured at 37°C in 5% humidified $CO_2$ in air and fed every 3 days with renewed culture medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% [vol/vol] newborn calf serum, 2 mM *L*-glutamine, 100 U/mL penicillin, 100 mg/mL streptokinase, 0.25 mg/mL fungizone, and 50 mg/mL gentamicin; all obtained from "Gibco Life Technologies" (Shanghai, China). For all of the assays cultured in monolayers HTF were trypticaly digested, pelleted, and counted using a hemocytometer. Cells between passages three and six were used for the following experiments.

**Fibroblast proliferation assay.** To investigate the effect of TGF-β1and α-MSH on fibroblast proliferation, several experimental groups were set up with different treatments as follows. In the control group, HTF were cultured in the regular culture medium; in the TGF-β1 group, HTF were cultured in the regular culture medium supplemented with $10^{-12}$ M TGF-β1 (our cell culture conditions were the same as those described in Cordeiro's article [25], in which TGF-β1 showed its peak activity on HTF proliferation at a concentration of $10^{-12}$ M); in the TGF-β1/α-MSH group, HTF were cultured in the regular culture medium supplemented with $10^{-12}$ M TGF-β1 and α-MSH at different concentrations ($10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ M). All *in vitro* experiments were performed in triplicate in 96-well tissue culture plates at an initial density of $5.0 \times 10^3$ cells per well. Cell proliferation was assessed 48 h after administration using a proliferation assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; "Promega", USA).

**Real-time reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted from $1 \times 10^5$ to $2 \times 10^5$ HTF using the RNeasy Micro Kit ("Qiagen Inc." USA) according to the manufacturer's protocol. The yield and purity of the RNA were spectrophotometrically determined, and the cDNA was prepared using the ReverAidTM First Strand cDNA Synthesis Kit ("Fermentas Inc.", USA). A real-time RT-PCR procedure was conducted according to the manufacturer's protocol for the SYBR® Premix Ex TaqTM Kit ("Takara Biotechnol." Japan). Reaction participants were assembled in a 96 well optical reaction plate ("Applied Biosystems", USA), and each well contained SYBR® Premix Ex TaqTM(2X), 200 nM of the forward primer, 200 nM of the reverse primer, ROX Reference Dye (50X), and the cDNA solution with a total volume of 20 μl. The expression levels of type I ($\alpha_1$) collagen, fibronectin, tumor necrosos factor α (TNF-α), intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), MMP-1, MMP-2, TIMP-1, and TIMP-2 mRNA were analyzed using the RT-PCR technique. The primers used for the target genes and β-actin can be found in table. The PCR was started at 95°C for 10 min (hot start) to activate the AmpliTaq polymerase, followed by 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, and plate reading at 60°C for 10 s). The reactions were performed using an ABI Prism 7000 Sequence Detection System ("Applied Biosystems"). The threshold cycle (*C*t) values were determined by the ABI Prism 7000 Software ("Applied Biosystems") and were normalized by subtracting the *C*t β-actin values. All experiments were performed in triplicate, and the relative amount of mRNA of each sample in individual experiments was calculated using the 2-Δ*C*t method [26].

**Statistical analysis.** All data is presented as means ± ± standard deviation (SD). The statistical analyses were conducted using SPSS version 10.0. Statistical analysis was performed using the Student's *t*-test. Probability (*P*) of less than 0.05 was considered significant. "Fold change" was used to show the results of mRNA expression. The fold changes of mRNA expression in the TGF-β1 and TGF-β1/α-MSH groups were calculated using the value of mRNA expression

The primers of target genes and β-actin in real-time RT-PCR

| Gene | Primer | | Reference |
|---|---|---|---|
| | sense | anti-sense | |
| β-actin | ATTGGCAATGAGCGGTTCCGC | CTCCTGCTTGCTGATCCACATC | [27] |
| type I ($\alpha_1$) collagen | GACTGGTGAGACCTGCGTGTA | CGGATCTCGATCTCGTTG | [28] |
| Fibronectin | CCGTGGGCAACTCTGTC | TGCGGCAGTTGTCACAG | [29] |
| TNF-α | CCCCAGGGACCTCTCTCTAAT | GGTTTGCTACAACATGGGCTAC | [27] |
| ICAM-1 | GCTATGCCTTGTCCTCTTG | ATACACACACACACACACACGC | [30] |
| VCAM-1 | CAAATCCTTGATACTGCTCATC | TTGACTTCTTGCTCACAGC | [30] |
| MMP-1 | AGTGACTGGGAAACCAGATGCTGA | GCTCTTGGCAAATCTGGCCTGTAA | [31] |
| MMP-2 | ATAACCTGGATGCCGTCGT | AGGCACCCTTGAAGAAGTAGC | [32] |
| TIMP-1 | CACCCACAGACGGCCTTCTGCAAT | AGTGTAGGTCTTGGTGAAGCC | [33] |
| TIMP-2 | CTCGCTGGACGTTGGAGGAAAGAA | AGCCCATCTGGTACCTGTGGTTCA | [33] |

in their group divided by the mean value of mRNA expression in the control group. The fold change of the control group was equal to 1.

## RESULTS

### *α-MSH inhibited TGF-β1 induced human Tenon's capsule fibroblasts growth*

Addition of $10^{-12}$ M TGF-β1 induced a significant increase in HTF proliferation, as compared to the control group ($P < 0.001$, fig. 1). α-MSH inhibited the TGF-β1 induced proliferation of HTF in a dose-dependent manner when the concentration was lower than $10^{-5}$ M. The inhibition effect of α-MSH at $10^{-4}$ M seemed to be a bit weaker than at $10^{-5}$ M, but there was no significant difference in the cell numbers between the two groups ($P = 0.318$, fig. 1).

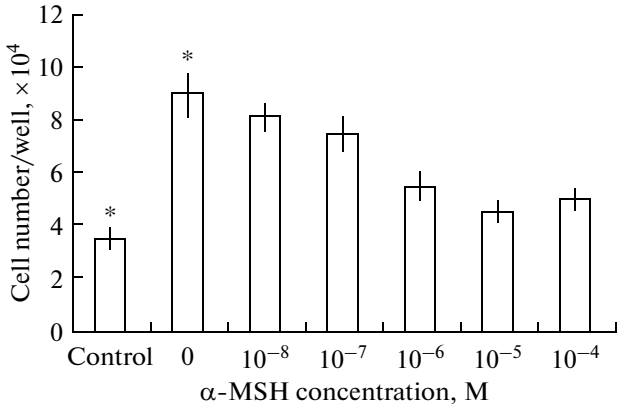


**Fig. 1.** Fibroblast proliferation after administration of TGF-β1 and α-MSH. Fibroblasts were cultured in DMEM in the control group, DMEM with $10^{-12}$ M TGF-β1 in the TGF-β1 group, and DMEM with $10^{-12}$ M TGF-β1 and α-MSH ranging from $10^{-8}$ to $10^{-4}$ M in the TGF-β1/α-MSH groups. *$P < 0.01$.

### *α-MSH attenuated fibrogenic, proinflammatory, and cell adhesion molecule gene expression induced by TGF-β1*

Expression levels of collagen α1 (type I), fibronectin, TNF-α, ICAM-1, and VCAM-1 mRNA were up-regulated in the TGF-β1 group as quantified by real-time RT-PCR (fig. 2). α-MSH significantly attenuated this up-regulation, however, α-MSH gene therapy did not affect fibronectin expression (TGF-β1 group vs. TGF-β1/α-MSH, $P = 0.181$) (fig. 2). In summary, α-MSH effectively suppressed fibrogenic, proinflammatory, and cell adhesion molecule gene responses after TGF-β1 administration.

### *α-MSH increased MMP activity in HTF*

Real time RT-PCR showed that MMP-1 mRNA expression was inhibited in HTF and MMP-2 mRNA expression was not altered by TGF-β1 administration, while α-MSH administration significantly up-regulated both MMP-1 and MMP-2 mRNA expression (fig. 2).

### *α-MSH attenuated TIMP activation in HTF*

The expression of TIMP in HTF was also evaluated using real time RT-PCR. The results indicated that TIMP-1 and TIMP-2 mRNA were significantly up-regulated after TGF-β1 administration, and α-MSH significantly attenuated this increment (fig. 2).

## DISCUSSION

TGF-β has been identified as an important component in wound healing, particularly in the conjunctival scarring response [34]. It was reported by Cordeiro et al. [25] that TGF-β1 stimulated the proliferation of HTF with a characteristic concentration-dependent response, with peak activities at $10^{-12}$. M In

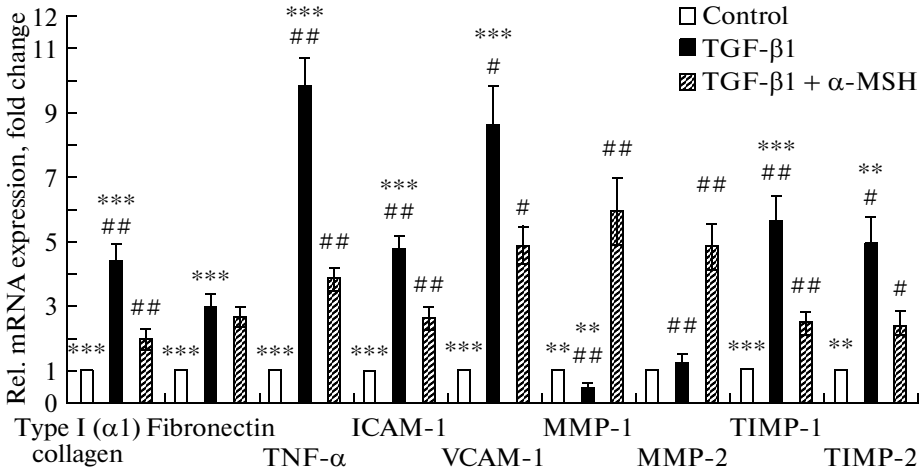


**Fig. 2.** Modulation effects of α-MSH on type I (α1) collagen, fibronectin, TNF-α, ICAM-1, VCAM-1, MMP-1, MMP-2, TIMP-1 and TIMP-2 mRNA expression stimulated by TGF-β1 (*$P$<0.05, **$P$<0.01, ***$P$<0.001, control group vs. TGF-β1 group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, TGF-β1 group vs. TGF-β1 + α-MSH group).

our study, we used TGF-β1 at a concentration of $10^{-12}$ M to induce proliferation of HTF in the same cell culture condition as Cordeiro's, and we found that α-MSH could inhibit the proliferation of HTF induced by TGF-β1 with a characteristic concentration-dependent response, with peak activities at $10^{-5}$ M. But in the following series experiments, we chose α-MSH at a concentration of $10^{-6}$ M, based on the HTF inhibitory effect of α-MSH in our results and previous studies [19, 35], in which α-MSH at $10^{-6}$ M showed good suppression on collagen production of human dermal fibroblasts.

Our results showed that α-MSH had an inhibitory effect on the expression of type I ($\alpha_1$) collagen mRNA of HTF, and no influence on the expression of fibronectin mRNA as described in the previous study [36]. However, there were paradoxical results in the previous studies about the effect of α-MSH on collagen mRNA expression of fibroblasts. Kokot's study demonstrated that α-MSH suppressed collagen production and mRNA expression in human dermal fibroblasts, which were up-regulated by bleomycin stimulation [35]. And Bohm's study showed a suppressive effect of α-MSH on collagen production in human dermal fibroblasts, but no effect on mRNA expression, which were both up-regulated by TGF-β1 stimulation, implying a posttranscriptional mechanism [19]. We considered that different cells and culture conditions could influence the effect of α-MSH on different steps of collagen synthesis.

Cell adhesion molecules are known as prognostic markers of fibrosis disease. Expression levels of ICAM-1 and VCAM-1, modulated by proinflammatory cytokines such as TNF-α, are up-regulated in idiopathic pulmonary fibrosis, carbon tetrachloride induced liver fibrosis, and fibrosis in chronic hepatitis C [3638]. A previous study reported that α-MSH attenuated lipopolysaccharide-induced vasculitis by down-regulating cell adhesion molecule expression [39]. In our study, α-MSH showed the same inhibitory effect on cell adhesion molecule expression in the course of HTF proliferation. Therefore, cell adhesion molecule modulation may play a role in the anti-fibrotic effects of α-MSH.

The imbalance between MMPs and TIMPs in the ECM contributes to the pathogenesis of fibrosis diseases. MMPs activity is regulated by TIMPs which bind to MMPs in a substrate and tissue-specific manner thus blocking their proteolytic activity [40]. It was reported that TGF-β mediated effects on fibroblast proliferation and collagen synthesis by modulating the MMP/TIMP system. MMP-1 expression decreased and TIMP-1 expression increased in human lung fibroblasts in response to TGF-β1 [41]. Decreased expression and activity of MMP-1, unaltered expression and activity of MMP-2 and increased levels of TIMPs were detected in TGF-β1 overexpressing transgenic mice [42]. In the presented study, we demonstrated a similar effect of TGF-β1 on the MMP/TIMP system in HTF and a reverse effect of α-MSH on the imbalance between MMPs and TIMPs. We speculated that matrix degradation manipulation of HTF might be involved in the anti-fibrotic effects of α-MSH. The rabbit conjunctiva after glaucoma filtration surgery showed altered transcript expression of MMPs and TIMPs [43]. Therefore, we hypothesized that α-MSH may reduce postoperative scarring after glaucoma filtration surgery by modulating the MMP/TIMP system of HTF.

Our findings on the suppressive activity of α-MSH on HTF proliferation and modulating effect of α-MSH on a series of correlative molecules in fibrosis finally highlight a novel biological activity that may be exploited in the treatment of conjunctival fibrosis dis-

order, such as bleb scarring after filtration surgery, and the hypothesis would be proved in our further studies *in vivo*.

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