Collagen metabolism - a novel target of the neuropeptide alpha-melanocyte-stimulating hormone

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Running title: alpha-MSH suppresses collagen synthesis

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Abstract

Suppression of collagen synthesis is a major therapeutic goal in treatment of fibrotic disorders. We show here that alpha-melanocyte-stimulating hormone (alpha-MSH), a neuropeptide well known for its pigment-inducing capacity, modulates collagen synthesis and deposition. Alpha-MSH in vitro suppresses the synthesis of collagen type I, III and V and down-regulates the secretion of procollagen type I C-terminal peptide (PICP) in human dermal fibroblasts treated with the fibrogenic cytokine transforming growth factor-beta1 (TGF-beta1). Alpha-MSH did not interfere with TGF-beta1 signaling since TGF-beta1-induced expression of collagen mRNA was not affected implying a posttranscriptional mechanism. Human dermal fibroblasts in vitro express high-affinity binding sites for MSH which were identified by RT-PCR and immunofluorescence analysis as the melanocortin-1 receptor (MC-1R). Immunohistochemical studies on normal adult human skin confirmed MC-1R expression in distinct dermal fibroblastic cells. The MC-1R on fibroblasts appears functionally relevant since alpha-MSH increased intracellular cAMP and coincubation with a synthetic peptide corresponding to human Agouti signaling protein abrogated the inhibition of TGF-beta1-induced PICP secretion by alpha-MSH. To assess the in vivo relevance of these findings, a mouse model was used in which dermal fibrosis was induced by repetitive intracutaneous injections with TGF-beta1. The inductive activity of TGF-beta1 on collagen deposition and number of dermal cells immunoreactive for vimentin and alpha-smooth muscle actin was significantly suppressed by injection of alpha-MSH. Melanocortins such as alpha-MSH may therefore represent a novel class of modulators with potential usefulness for treatment of fibrotic disorders.
**Introduction**

Fibrotic and sclerotic diseases comprise a large and heterogeneous group of inflammatory, idiopathic, toxic, hereditary and pharmacologically induced disorders such as hypertrophic scars, keloids, localized scleroderma, systemic sclerosis, sclerodermic graft-versus-host disease of the skin, cirrhosis of the liver, idiopathic and bleomycin-induced lung fibrosis or cyclosporine-induced nephropathy. The therapeutic options are limited and treatment of these disabling disorders is still a challenge.

A key feature of fibrotic disorders is excessive production of extracellular matrix, mainly type I collagen, followed by a gradual loss of organ function which, in some cases, can be fatal. In the last years it became apparent that transforming growth factor-beta\(_1\) (TGF-beta\(_1\))\(^1\), a multifunctional cytokine, is crucially involved in the pathogenesis of fibrotic disorders (1-5). It induces fibrosis by various ways (reviewed in ref. 6). It enhances the expression of several collagens including type I, III and V. TGF-beta\(_1\) decreases the production of matrix degrading proteases and enhances the synthesis of inhibitors of such proteases. TGF-beta\(_1\) also increases extracellular cross-linking of collagen by enhancing the expression and the activity of lysyl oxidase (7). These multiple activities explain the potent fibrotic effect of TGF-beta\(_1\). Therefore, strategies aiming at antagonizing the strong profibrotic effect of TGF-beta\(_1\) are regarded as a promising approach to prevent excessive collagen accumulation in fibrotic disorders (8-10).

Alpha-melanocyte-stimulating hormone (alpha-MSH) is a tridecapeptide generated from pro-opiomelanocortin (POMC) by proteolytic cleavage (reviewed in ref. 11). It was originally isolated from the pituitary gland and was characterized as a pigment-inducing factor regulating the coat color of many vertebrate species but turned out to regulate many other biological activities with regard to the skin (reviewed in ref. 11,12). The biological activities of alpha-MSH are mediated by a family of structurally related receptors that are known as the melanocortin receptors

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\(^{1}\text{Abbreviations: }\text{alpha-MSH, alpha-melanocyte-stimulating hormone; alpha-SMA, alpha-smooth muscle actin; ASIP, Agouti signaling protein; BSA, bovine serum albumin; CTGF, connective tissue growth factor; FCS, fetal calf serum; HDF, human dermal fibroblasts; MC-R, melanocortin receptor; MMP, matrix metalloproteinase; NDP-MSH, [Nle4, D-Phe7]alpha-MSH; PBS, phosphate buffered saline; PICP, procollagen I C-terminal peptide; POMC, pro-opiomelanocortin; TGF-beta\(_1\), transforming growth factor-beta\(_1\).}
(MC-Rs). They belong to the superfamily of G-protein-coupled receptors with seven transmembrane domains and they activate adenylate cyclase after ligand binding. Five MC-R subtypes have been cloned which differ in their relative affinities to alpha-MSH and the other melanocortins (13,14).

Here, we show that in addition to its multiple biological effects alpha-MSH suppresses TGF-beta_1-induced collagen synthesis by human dermal fibroblasts (HDF) in vitro. This effect is mediated via the MC-1R. Alpha-MSH exerts its antifibrogenic activity also in vivo since injection of alpha-MSH into mice reduces TGF-beta_1-induced fibrosis. Our data establish a role for melanocortins in fibroblast biology and point towards a therapeutic potential of alpha-MSH and its analogues in the treatment of fibrotic and sclerotic diseases.

**Materials and Methods**

**Cells and culture conditions.** HDF from neonatal foreskin and adult skin as well as normal human melanocytes were purchased from CellSystems, St. Katharinen, Germany. The human fibrosarcoma cell line HT-1080 was obtained from the American Tissue Cell Collection (ATCC). Fibroblasts were routinely cultured in RPMI 1640 (PAA, Cölbe, Germany), 1% Gln, 1% penicillin/streptomycin (both from PAA) and 10 % fetal calf serum (FCS) (Biochrom, Berlin, Germany) in a humidified atmosphere of 5% CO_2 at 37°C. Normal human melanocytes were cultured in MBM2 medium plus MGM-3 aliquots as indicated by the manufacturer (Clonetics, Walkersville, MD).

**RNA extraction, RT-PCR and sequencing.** Total RNA was isolated from cells using a commercial purification kit (Promega, Madison, WI). After DNA digestion, 1 µg of total RNA was reverse-transcribed with 15 U AMV-RT (Promega). The resulting cDNA was amplified with 2.5 U Taq polymerase (Promega) and MC-R primers under conditions identical to those described previously (15-17). Primer sequences and the sizes of their amplification products are given in
Table I. Only RNA samples that did not yield amplification products were reverse-transcribed. For some positive controls, genomic DNA from HDF was prepared by routine protocols. Amplicons were separated in 1.5 % agarose gels. The resulting MC-1R-related band in HDF was purified using a gel extraction kit (Qiagen, Santa Clarita, CA), cloned into pGEM-T easy vectors (Promega) and sequenced (4base lab GmbH, Reutlingen, Germany).

Quantitative real-time PCR. Quantification of mRNA levels of the various procollagen chains was carried out by real-time fluorescence detection as described previously (18). cDNA was prepared amplified by PCR in the ABI Prism 7700 sequence detector (PE Biosystem, Foster City, CL). Primer and probe sequences were designed by the Primer Express software (PE Biosystems) or were supplied by PE Biosystems (GAPDH) (17). COL(I)alpha: sense 5’-CAGCCGCTTCCACCTACAGC-3’, antisense 5’-AATCACTGTCTTGCCCCAGG, probe 5’ ACTGTCGATGGGCTGCA-CGAGTCAC-AC-3’. COL(I)alpha: sense 5’-GATTGAGACCCTTCTTACTCCTGAA-3’, antisense 5’-GGGTGGCTGAGTCTCAAGTCA-3’, probe 5’-TCTAGAAAGAACCCAGCTCGC-ACATGC-3’. COL(III)alpha: sense 5’-TCCAACGTCTCCTACTCGCC-3’, antisense 5’-GAGGG-CCTGGATCTCCCTT, probe 5’-CTAATGGTCAAGGCCGCAGGACCTCAAGGCC. Probes were labeled at the 5’ end with the reporter dyes 6-carboxyfluorescein or VIC and at the 3’ end with the quencher dye 6-carboxy-tetramethyl-rhodamine. The 5’-nuclease activity of the Taq polymerase (Applied Biosystems, Germany) cleaved the probe and released the fluorescent dyes which were detected by the laser detector of the sequence detector. After the detection threshold was reached, the fluorescence signal was proportional to the amount of PCR product generated. The initial template concentration could be calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR. Relative gene expression levels were calculated using standard curves generated by serial dilutions of cDNA from HT1080 cells. The relative amounts of gene expression were calculated by using the expression of GAPDH as an
internal standard. Expression of each gene was assessed by three independent PCR analyses and calculation of the mean ± SEM. Data were analyzed by the Student’s t-test.

**Binding studies.** [Nle4, D-Phe7]alpha-MSH (NDP-MSH) (Bachem, Bubendorf, Switzerland) was radio-iodinated by the chloramine T method and purified by HPLC. Binding studies were performed as described previously (19). In short, cells were washed with binding buffer and distributed into 96 well plates. Cells were then incubated for 2 hrs at 37°C with 50 µl binding buffer in each well containing a constant concentration of 0.2 nM of [125I]-NDP-MSH and appropriate concentrations of unlabelled ligand. After incubation, the cells were washed with 0.2 ml of ice-cold binding buffer, detached with 0.2 ml 0.1 N NaOH and the radioactivity was counted in each well. The binding assays were performed in duplicate wells. Radioactivity was determined by a gamma counter (Wallac, Wizard automatic) and data were analyzed with a software package for radioligand binding analyses. Data were analyzed by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modeling.

**Immunofluorescence.** HDF were seeded into chamber slides and were fixed with methanol for 30 min at -20°C or, for surface staining, with 4 % paraformaldehyde for 30 min at room temperature. Non-specific binding was blocked with 5% goat/donkey serum for 1 hr at room temperature. Cells were then incubated for 1 hr with a rabbit polyclonal antibody against the human MC-1R (1 µg/ml). Production and characterization of the anti-human MC-1R is described in detail elsewhere (20,21). In some experiments double staining with a monoclonal antibody against protein disulfide isomerase (1:100; Dako, Hamburg, Germany), a cytoplasmic marker (22,23), was performed. Bound antibodies were visualized with a donkey anti-rabbit antibody coupled to Texas Red (1:100, Dianova, Hamburg, Germany) and with a goat anti-mouse antibody coupled to fluorescein isothiocyanate (1:100, Dako). After mounting, specimens were examined with a confocal laser-scanning microscope (TCS E, LEICA, Heidelberg, Germany).
**Determination of cAMP.** For intracellular cAMP measurements, 2 x 10^4 HDF were seeded into 96-well tissue culture plates. On the next day the routine culture medium was changed to RPMI 1640 containing 1% FCS. Cells were cultured for additional 24 hrs followed by stimulation with alpha-MSH as indicated for 20 min in presence of 0.1 mM isobutyl methylxanthine. 0.1-5 µM forskolin was used as a positive control. After incubation, supernatants were removed and cells were lysed. cAMP levels in the lysates were determined by a specific enzyme immunoassay according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Freiburg, Germany). Triplicate wells were used for each individual treatment and statistical analysis was performed using the Student’s $t$-test.

**Collagen analysis.** HDF were seeded into 6-well tissue culture plates (250 000 per well) and allowed to attach and grow for 16 hrs. Subconfluent cell monolayers were then incubated with minimal essential medium containing 0.5% FCS and 50 µg/ml L-ascorbic acid for 24 hrs in the presence of TGF-beta1 (10 ng/ml), alpha-MSH (10^{-6} M) or a combination of both substances. HDF were rinsed and depleted for 1 hr in Met/Cys-free minimal essential medium (ICN Costa Mesa, CA). Cells were labelled with [^{35}S]-Met/Cys mix (50 µCi/ml) for 16 hrs in the presence of ascorbate and the above agents. Media were collected, cell cultures rinsed three times with ice-cold TBS and cells scraped into 1% NP-40 in TBS with a rubber policeman. Cell lysates were centrifuged and aliquots of the supernatant subjected to liquid scintillation counting to determine cell mass. Media and combined cell lysates/scrapings were treated with pepsin to destroy non-collagenous proteins (24). Proteins in either fraction were precipitated using MeOH/chloroform and processed for SDS-PAGE (5% acrylamide; acryl:bisacryl = 37.5:1). All loaded aliquots were calibrated for cell mass, so that all slots contained pepsin treated collagen derived from the same amount of cells. Slab gels were fixed and radiolabelled collagens were detected by autoradiography.
(25). Representative gels were subjected to densitometry using the Biostep Phoretix Grabber (Jahnsdorf, Germany)

**Determination of procollagen I C-terminal-peptide.** Amounts of procollagen I C-terminal peptide as a marker for procollagen I secretion were determined using a commercially available ELISA (TaKaRa, Shiga, Japan). HDF were seeded into 12-well tissue culture plates at a density of 250,000 cells per well. Confluent HDF were then deprived for 2 days from FCS and were subsequently stimulated with alpha-MSH (10⁻⁶ M to 10⁻¹⁰ M), TGF-beta₁ (10 ng/ml) or both agents in presence of 50 µg/ml ascorbate. In some experiments cells were coincubated with a synthetic peptide corresponding to the amino acids 87-132 of human Agouti signaling peptide (Phoenix Pharmaceuticals Belmont, CA) at a tenfold molar excess. Culture supernatant were harvested after 48 hrs, centrifuged and frozen at -70°C until use. Statistical evaluation from triplicate wells was performed using the Student’s t-test.

**Mouse model for cutaneous fibrosis.** For *in vivo* evaluation of the antifibrogenic effect of alpha-MSH, a mouse model previously described by Shinozaki et al. (26) was used with slight modifications. Accordingly, cutaneous fibrosis was induced by intracutaneous injections of 800 ng TGF-beta₁ into the neck of new-born Balb/c mice on three consecutive days. Treatment groups (4 groups of 3 mice each) consisted of mice injected with TGF-beta₁, alpha-MSH (25 µg), TGF-beta₁ plus alpha-MSH and the solvent (0.1% BSA in PBS) in which TGF-beta₁ had been solubilised. On day 4, mice were sacrificed and 4 mm punch biopsies were taken from the sites of injection for immunohistochemical analysis.

**Immunohistochemistry.** After fixation in 4% paraformaldehyde and paraffin-embedding biopsies from mouse skin were processed for hematoxylin and eosin (H & E) stain, for van Gieson stain in which collagen appears red, and for recorcin-fuchsin stain according to Weigert in which elastic tissue appears black. For collagen staining sections were treated with 1 mg/ml pepsin
(Sigma, St. Louis, MO) in 0.5 M acetic acid, washed and incubated with a rabbit antibody against collagen type I (1:100; DPC Biermann, Bad Nauheim) for 1 hr. For staining of vimentin sections were microwave-treated to unmask epitopes followed by incubation with a polyclonal antibody from Abcam, Cambridge, UK, for 30 min at 37°C. For staining of alpha-smooth muscle actin, a monoclonal antibody from Dunn Lab., Asbach, Germany, was incubated for 1 hr at 2 µg/ml without prior demasking. Immunohistochemistry for MC-1R in sections of normal adult human skin (n>5) was performed exactly as outlined before (17,21). Sections were developed by the indirect immunoperoxidase technique using 3-amino-9-ethylcarbazole (Sigma) as a chromogen. Negative controls included incubation with control IgG at the same protein concentration as the primary antibody, omission of the first antibody, or pre-incubation with the immunogenic peptide in tenfold weight excess in case of MC-1R immunostaining. Vimentin and alpha-smooth muscle actin immunostaining in sections of mouse skin was quantitatively assessed by counting the number of immunoreactive interfollicular dermal cells in 3 high-power fields (x400). Means ± SD from 3x4 independent experiments were analyzed by ANOVA.

Results

Alpha-MSH modulates collagen expression by HDF in vitro. We addressed the question if alpha-MSH can modulate the key function of HDF, namely expression and secretion of collagen. To this end, cultured normal HDF from neonatal foreskin were treated with alpha-MSH, TGF-beta\textsubscript{1} or both substances. The amounts of collagen present in cell lysates and in culture supernatants were separately determined after metabolic labelling, pepsin digestion and SDS-PAGE.

TGF-beta\textsubscript{1}, a well known inducer of collagen synthesis (6), increased the amount of secreted collagens in the culture medium (Fig. 1A). Alpha-MSH alone appeared to reduce the extracellular amount of collagens I, III and V by 50-70 % as determined by densitometry (Fig. 1A) (and data not shown). This reduction was not due to intracellular retention, although there was some increase in alpha\textsubscript{1}(I) chains which we attribute to a higher synthesis rate. The intracellular bands showed no
delayed migration and thus excluded significant posttranslational overmodification due to abnormal intraendoplasmatic retention (Fig. 1A). Most strikingly, alpha-MSH dramatically reversed the stimulatory effects of TGF-beta on the extracellular collagen presence with the strongest effects on collagens I and III and somewhat milder effects on collagen V (Fig. 1A). In the conspicuous absence of intracellular retention these findings implicate either intracellular or extracellular proteolytic degradation or a combination of both.

To further substantiate the activity of alpha-MSH on collagen synthesis and/or secretion, we determined the amount of procollagen I C-terminal peptide (PICP) in culture media of HDF stimulated with alpha-MSH, TGF-beta, or both agents. The addition of TGF-beta led to a dramatic increase of PICP in the culture medium by more than 500%. In accordance with the modulatory effect of alpha-MSH on the TGF-beta-induced collagen biosynthesis and subsequent secretion, we found significantly reduced secreted amounts of PICP by HDF (677.9±60.1 pg/ml versus 1313.9±136 pg/ml; p<0.005) (Fig. 1B). Alpha-MSH alone, in contrast, did not affect the basal amounts of secreted PICP. These findings suggested either an intracellular or extracellular cause for the reduction of secreted procollagen I.

Modulation of collagen synthesis by alpha-MSH is not mediated by reduced mRNA expression. We next wondered if the modulatory activity of alpha-MSH on collagen synthesis is regulated at the transcriptional level. HDF from neonatal foreskin were stimulated with alpha-MSH, TGF-beta, or both agents for 12 hrs. The relative mRNA levels for the alpha1(I) and alpha2(I) chains of collagen I (alleles COL1A1 and COL1A2, respectively) and for the alpha1(III) chains for collagen III (alleles COL3A1) were subsequently determined by quantitative real-time PCR. TGF-beta significantly increased the mRNA levels of collagen type I alpha1 and alpha2 as well as that of collagen type III alpha1 as compared to non-treated cells (Table II). The observed rate of increase in the amount of these collagens by TGF-beta was in accordance with earlier reports (27). Despite some variation neither alpha-MSH alone nor coincubation of alpha-MSH and
TGF-beta1 caused significant reduction in the relative levels of the collagen mRNA’s (Table II). Similar results were obtained when HDF were treated with TGF-beta1 and alpha-MSH for 24 hrs (data not shown). These findings show that alpha-MSH does not interfere with TGF-beta1 signaling and that alpha-MSH may affect collagen expression at the posttranscriptional level.

Detection of high-affinity binding sites for MSH on HDF. The identified effects of alpha-MSH on the amount of extracellular collagen suggested the presence of specific binding sites in HDF. Therefore, we examined HDF from neonatal foreskin for competitive radioligand binding using an iodinated synthetic alpha-MSH analogue, Nle4, D-Phe7]alpha-MSH (NDP-MSH). Displacement was performed with unlabelled ligand at varying concentrations and COS-1 cells were used as a negative control. HDF exhibited a specific and saturable binding kinetic with [125I]NDP-MSH. The affinity of the radioligand was similar to COS-1 cells transfected with the human MC-1R (Fig. 2). The Ki values were 0.058±0.012 nM for the HDF and 0.086±0.033 nM for COS-1 cells transfected with the human MC-1R, the latter value being similar to previous studies (19). HDF therefore exhibited similar affinity but slightly lower expression levels of high-affinity MSH binding sites than COS-1 cells transfected with MC-1R. These data strongly suggested that alpha-MSH binds to specific surface receptors on the surface of HDF which appear to mediate its biological action.

Expression of MC-1R in HDF in vitro and in situ. To investigate in detail the expression of MC-Rs in HDF, we performed RT-PCR analysis using primers against all known MC-Rs (Table I). MC-1R was the only MC-R expressed in HDF derived from neonatal foreskin (Fig. 3A). Similarly, HDF derived from adult human skin expressed MC-1R at the RNA level (data not shown). The MC-1R amplification product of HDF comigrated exactly with that of normal human melanocytes used as a positive control (Fig. 3A). The identity of the amplification product in HDF (416 bp) was determined by DNA sequencing and found to be identical with the mRNA sequence of MC-1R as
deposited in the NCBI (Table I, and data not shown). In contrast to MC-1R, no other MC-R was expressed in HDF as shown by RT-PCR (Fig. 3A). The amplification products of the positive controls were all of the expected size (Fig. 3A and Table I) (15-17).

To examine the expression of the MC-1R at the protein level in HDF in vitro we next performed immunofluorescence studies. For melanoma cells in culture it has been reported that binding sites for MSH are confined to certain areas on the cell surface (17,28) while in other cutaneous cell types MC-1R immunoreactivity appeared more randomly distributed (17). Immunofluorescence studies with HDF fixed either in paraformaldehyde (data no shown) or in methanol gave similar images. MC-1R antigenicity was visible as a characteristic punctate staining randomly distributed with accentuation in the cell periphery (Fig. 3B). Control experiments with pre-immune serum or neutralization with the immunogenic peptide used for generation of the anti-MC-1R antibody did not produce any staining (Fig. 3B).

To check if expression of MC-1R is maintained in HDF in situ, skin sections of normal adult human skin were processed for immunohistochemistry. MC-1R immunoreactivity was absent in interfollicular dermal fibroblasts at the light microscopic level. However, in distinct fibroblastic cells of the connective tissue sheath of the hair follicles MC-1R immunoreactivity was consistently detectable (Fig. 3C). MC-1R immunostaining in these cells had a punctate pattern and was localized mainly in the cytoplasm (Fig. 3C). Immunostaining with an antibody against vimentin confirmed the nature of these cells as fibroblasts (data not shown). Pre-incubation with the antigenic peptide or pre-immune serum, in contrast, did not produce any staining (Fig. 3C). As reported previously (21), MC-1R immunoreactivity was also detected in distinct epithelia of the skin appendages, for example of the outer root sheath hair follicle keratinocytes (Fig. 3C). Collectively, these studies data demonstrate that MC-1R expression is not restricted to HDF in culture but is also detectable in situ in distinct dermal fibroblast populations of normal human skin.

Functional coupling of MC-1R expressed in HDF. To investigate functioning of the identified MC-1R on HDF we performed cAMP measurements of cells stimulated with varying
doses of alpha-MSH. It has been previously shown that all members of the MC-R family are G protein-coupled receptors whose interaction with the ligand result in stimulation of adenylate cyclase. Alpha-MSH increased the amount of intracellular cAMP in a dose-dependent manner as compared to non-treated cells (Fig. 4). This effect was maximal at 10^{-6} M (p<0.05) and similar to stimulation of HDF with 0.1 µM forskolin. Concentrations of alpha-MSH higher than 10^{-9} M did not lead to significant changes in the amount of intracellular cAMP as compared to non-stimulated HDF (Fig. 4).

To clarify if MC-1R mediates the inhibitory action of alpha-MSH on TGF-beta1-induced collagen synthesis in HDF, we performed blocking experiments with a synthetic peptide corresponding to the amino acids 87-132 of human Agouti signaling peptide (ASIP), a natural and highly potent antagonist of MC-1R but also of MC-4R (29). Cys-rich C-terminal ASIP fragments were previously shown to be as potent as full-length ASIP (30). Since HDF express only MC-1R (Fig. 3A) we hypothesized that the synthetic ASIP peptide would block the antagonistic effect of alpha-MSH on collagen synthesis induced by TGF-beta1. Coincubation of the synthetic ASIP fragment at 10^{-7} M plus alpha-MSH at 10^{-8} M and TGF-beta1 (10 ng/ml) completely abrogated the antagonistic effect of alpha-MSH on PICP secretion (Fig. 5). In contrast, the synthetic ASIP fragment alone did not affect secretion of PICP in a significant manner (Fig. 5). As outlined above, alpha-MSH alone (Fig. 1B), or coincubation of TGF-beta1 plus the synthetic ASIP did not exert any modulatory effect on PICP secretion (data not shown). Taken together, these data strongly support the concept that alpha-MSH via acting on MC-1R modulates fibroblast activity, or collagen synthesis, respectively.

Alpha-MSH has antifibrogenic activity in vivo. We next wished to know if alpha-MSH can also modulate collagen synthesis and secretion in vivo. Therefore, we employed an animal model in which cutaneous fibrosis is elicited by repetitive intracutaneous injections of high doses of TGF-beta1 (26). We chose newborn mice as they contain significantly less collagen in their skin than
Adult mice thus rendering the former suitable for evaluation of fibrogenic and antifibrogenic stimuli. Accordingly, newborn mice were injected into the neck for three consecutive days with TGF-beta1 (800 ng), alpha-MSH (25 µg), alpha-MSH plus TGF-beta1, or PBS. On day 4 punch biopsies were taken from the injection sites and subjected to biochemical and histological analysis. As contrasted to samples from adult murine skin, the hydroxy-proline content in the newborn mice samples was below the detection limit to allow collagen analysis by this approach (data not shown). Therefore, we used semiquantitative histochemical and immunohistochemical analysis to assess the effect of alpha-MSH on TGF-beta1-induced skin fibrosis. When compared to PBS, injections with TGF-beta1 induced dermal thickening and fibrosis as well as increased numbers of collagen fibers as shown by H & E and Van Gieson stains as well by immunohistochemistry using an anti-collagen type I antibody (Fig. 6A,D,G versus B,E,H). Elastic fibers in the skin of untreated mice, in contrast, were sparse and were detected primarily in the dermal vasculature with no increase in upon injection with TGF-beta1 (data not shown). Injections with alpha-MSH alone did not produce any changes as compared to mice treated with PBS/BSA (data not shown). On the other hand, coinjection of mice with TGF-beta1 plus alpha-MSH resulted in a significant reduction in the amount of extracellularly deposited collagen as compared to mice injected with TGF-beta1 alone (Fig. 6C,F,I versus B,E,H). To further corroborate the antifibrogenic activity of alpha-MSH in vivo we examined the in situ number of dermal cells immunoreactive for vimentin, an established fibroblast marker, as well as for alpha-smooth muscle actin (SMA), a fibroblast activation and myofibroblast transdifferentiation marker. It has been previously shown that alpha-SMA is strongly induced in fibroblasts by TGF-beta1 in vitro (31). Enhanced in situ expression of alpha-SMA has also been associated with increased tissue injury and progression of interstitial fibrosis (32-34). As compared to mice injected with PBS/BSA, TGF-beta1 significantly increased the number of dermal cells immunoreactive for both vimentin and SMA. This effect was strongly antagonized by coinjection of TGF-beta1 plus alpha-MSH (Table III; p<0.001 for vimentin; p<0.02 for alpha-SMA). In accordance with the above data, alpha-MSH alone did not have any modulatory activity
Alpha-MSH suppresses collagen synthesis on the number of vimentin or alpha-SMA positive cells in murine skin (data not shown). These findings demonstrate that the modulatory activity of alpha-MSH on TGF-beta_1-induced collagen synthesis and deposition is not confined to HDF in vitro but is also operational in vivo.

Discussion

We have shown here that the neuropeptide alpha-MSH antagonizes the action of TGF-beta_1 on collagen synthesis in HDF in vitro as well as in a mouse model of cutaneous fibrosis in vivo. This represents an analogy to the antagonistic action of this neuropeptide on IL-1-mediated responses which appears to be affected by suppression of NF-KappaB activation (35). It has been previously shown that the TGF-beta_1-mediated effect on type I procollagen alpha_1 transcription in rat kidney fibroblasts depends on the expression of connective tissue growth factor (CTGF), a downstream target of TGF-beta_1 and which is negatively regulated by cAMP (36). Since alpha-MSH increases intracellular cAMP we originally hypothesized that treatment with alpha-MSH would lead to reduced CTGF expression and, consequently, to reduced mRNA expression of collagen type I and III. However, the lack of any effect of alpha-MSH on the mRNA levels of type I and III collagens precludes an interference of alpha-MSH with the signal transduction of TGF-beta_1. The latter conclusion is in accordance with findings showing that alpha-MSH in HDF does neither inhibit TGF-beta_1-induced phosphorylation nor block the nuclear translocation of Smad2/3 (data not shown). It is possible that the observed differences between the aforementioned findings and our data are due to cell-specific differences (i.e., HDF versus rat kidney fibroblasts) or differences in the individual experimental setting (e.g., the use of artificial cAMP versus natural cAMP inducers).

The fact that alpha-MSH does not interfere with TGF-beta_1-mediated transcription of collagen type I suggests a posttranscriptional mechanism for the suppressive effect on collagen synthesis, e.g., translational repression or enhanced extracellular proteolytic degradation. Regarding the latter, alpha-MSH may suppress the activity of the C- and N-terminal procollagen
proteinases which remove the propeptides from secreted procollagen. This would lead to a reduced formation of collagen fibers. Alpha-MSH may also stimulate degradation of secreted procollagen by activating members of the matrix metalloproteinase family, for example MMP-1 (37) and/or other members such as MMP2 and MMP9. Since C- and N-proteinases are only marginally active in fibroblast cell cultures (38) the observed reduction of collagens in the culture medium may point to extracellular degradation of single procollagen trimers. In addition to the above potential mechanisms, it is possible that alpha-MSH affects the intracellular free pool of selected amino acids required for collagen synthesis. Recently, it has been demonstrated that selected cellular environmental changes such as hypoxia inhibits proline uptake while leaving methionine uptake relatively unaffected (39). With regard to alpha-MSH, however, nothing is known about a potential influence on uptake and transport of amino acids. Further studies are thus necessary to elucidate the molecular mechanism by which alpha-MSH modulates collagen synthesis.

The presence of MC-1R in HDF as shown by radioligand binding, RT-PCR and immunofluorescence in this paper explains a number of previously reported activities of alpha-MSH on human fibroblasts. It was reported that alpha-MSH can block the IL-1-induced production of prostaglandin E in a lung fibroblast cell line (40). We have shown that alpha-MSH in vitro increases the secretion of IL-8 and modulates the activation of the transcription factors NF-KappaB and AP-1 in HDF (41).

The inhibitory action of alpha-MSH on TGF-beta1-induced collagen synthesis and/or secretion by HDF in vitro and its antifibrogenic activity in vivo adds another dimension to the broad spectrum of biological activities of this neuropeptide. The skin itself contains the full capacity to produce POMC peptides (42) and HDF in vitro have recently been shown generate immunoreactive amounts of adrenocorticotropin and alpha-MSH (43). However, transgenic mice with a signaling-deficient MC-1R are not sclerodermic (44). In light of our findings it is puzzling that peripheral blood levels of POMC peptides are elevated in patients with systemic sclerosis (45), and increased in situ expression of POMC has been detected in inflammatory cells of keloids (46).
It remains to be determined whether these findings are part of the cutaneous stress response (11) or are related to involvement of alpha-MSH in collagen metabolism. Since the amounts of alpha-MSH used in our studies were higher than the plasma concentration of this neuropeptide in man, the exact role of alpha-MSH in fibroblasts under physiologic and pathophysiologic conditions requires further investigation.

Our findings on the modulating activity of alpha-MSH on collagen synthesis finally highlight a novel biological activity that may be exploited in the treatment of fibrotic disorders. alpha-MSH is a small molecule with a molecular weight of 1.66 kDa. Preliminary data have been shown that nickel-induced contact dermatitis in human can be suppressed by a topical alpha-MSH (100 µM) (11). It is known that systemic or intradermal injection of alpha-MSH or its analogue NDP-MSH into humans is well tolerated and has little toxicity (47,48). The latter alpha-MSH derivative was found to be 10-1000-fold active than alpha-MSH depending on the applied bioassay (49). In the past, a variety of alpha-MSH analogues with increased potency and prolonged activity have been synthesized (49). These bioactive peptides include minimal fragment analogues of alpha-MSH containing the core sequence 6-9 and 7-9. The truncated alpha-MSH peptides are active at µM concentration and are MC-R subtype-specific. The low molecular weight of such peptide fragments may render them suitable for transdermal delivery in vivo.

**Acknowledgement**

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    2236
    286
    *Peptides* **4**, 813-816
Legends

Fig. 1

Alpha-MSH suppresses collagen synthesis and secretion by HDF in vitro. (A) Confluent cells were stimulated with 10 ng/ml TGF-beta (T), 10^{-6} M alpha-MSH (M), both substances (T+M), or were left untreated (N/A) for 24 hrs. After metabolic labeling with \([^{35}\text{S}]-\text{Met}\), proteins in culture supernatants and cell lysates were digested by pepsin and separated by 5% SDS-PAGE followed by autoradiography. (B) Confluent HDF were stimulated for 48 hrs with the indicated agents at concentration as described above. Immunoreactive amounts of PICP in the culture media were determined by ELISA. Data represent means ± SEM from three independent experiments.

Fig. 2

Detection of high-affinity binding sites for MSH on HDF in vitro as shown by radioligand binding. Cells were examined for competitive binding with 0.2 nM of the synthetic alpha-MSH analogue \([^{125}\text{I}]-\text{NDP-MSH}\) and unlabelled ligand. Binding affinity and expression levels of HDF (squares) were compared with COS-1 cells transfected with the human MC-1R (circles) and with non-transfected COS-1 cells (triangles). Data are a representative set of several experiments with similar results.

Fig. 3

Expression of the MC-1R in HDF in vitro and in situ. (A) RT-PCR analysis of total RNA derived from cultured HDF using specific primers against the 5 MC-Rs. Negative controls (NC) consisted of a genomic contamination control using total RNA without reverse transcription and a reaction mixture control using H_2O instead of the template. Normal human melanocytes (NHM) and genomic DNA from HDF were used as positive controls (PC). (B) MC-1R immunoreactivity in cultured HDF as shown by double immunofluorescence. Cells were stained with a polyclonal MC-1R antibody (red signal) and a monoclonal against protein disulfide isomerase (green signal), a
cytoplasmic marker. Scale bar = 20 µm. (C) MC-1R immunohistochemistry of normal adult human skin using a polyclonal anti-MC-1R antibody and the immunoperoxidase technique. MC-1R immunoreactive fibroblasts were detected within the connective tissue sheath of the hair follicle (arrows) but not in the negative control (pre-incubation with the immunogenic peptide). *MC-1R immunoreactivity was also detected in outer root sheath epithelia. Upper panel, magnification: x100. MC-1R immunoreactivity in perifollicular fibroblasts displayed a punctate staining pattern. Lower picture, magnification: x200.

Fig. 4

**Alpha-MSH induces intracellular cAMP in HDF.** Cells were stimulated with alpha-MSH at varying doses for 20 min or were left untreated (N/A). As a positive control, cells were treated with 0.1 µM forskolin (FSK). After cell lysis, intracellular AMP levels were determined by an enzyme-linked immunoassay. Data represent the means ± SEM from triplicate experiments.

Fig. 5

**Alpha-MSH-mediated inhibition of PICP secretion induced by TGF-beta_1 is blocked by ASIP.** Confluent HDF were left untreated (N/A) or were stimulated for 48 hrs with 10^{-8} M alpha-MSH (M), 10 ng/ml TGF-beta_1 (T) or both substances (T+M). Competitive blocking was performed by incubating the cells at 10^{-7} M with a synthetic peptide fragment (A) corresponding to the Cys-rich C-terminal domain (aa 87-132) of human ASIP. Immunoreactive amounts of PICP in the culture media were determined by ELISA. Data represent means ± SD from one representative experiment which was reproduced twice with identical results.

Fig. 6

**Alpha-MSH has antifibrogenic activity in vivo.** Cutaneous fibrosis in newborn mice was induced by repetitive TGF-beta_1 injections. Mice were injected on three consecutive days into the neck intracutaneously with PBS/BSA (A,D,G), 800 ng TGF-beta_1 (B,E,H), or TGF-beta_1 plus 25 µg
alpha-MSH (C,F,I). On day 4, mice were sacrificed and 4 mm punch biopsies were taken from the sites of injection. Biopsies were fixed, embedded in paraffin and processed for H & E staining (A-C), Van Gieson staining (D-E) in which collagen fibers appear red, and for immuno-histochemistry using an antibody against collagen type I (G-I) in which bound antibodies were visualized by the immunoperoxidase technique (red immunoreactivity). Magnification: 200x. Figures are representative sets of three independent experiments with similar results.
Table I. Primer sets used for RT-PCR analysis of MC-Rs

<table>
<thead>
<tr>
<th>Gene accession number</th>
<th>Gene product</th>
<th>Primer (F, forward; B, backward)</th>
<th>Size†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1R/AF326275</td>
<td>MC-1R</td>
<td>F: 5’-GCCACCATCGCCAAGAACC-3’ B: 5’-ATAGCCAGGAAGAAGACCA-3’</td>
<td>416</td>
<td>15,17</td>
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<tr>
<td>MC2/Z25470‡</td>
<td>MC-2R</td>
<td>F: 5’-CTGCATTITTCCTGGATCT-3’ B: 5’-AAGCTGACATGGATGC-3’</td>
<td>380</td>
<td>15,17</td>
</tr>
<tr>
<td>MC3/NM019888</td>
<td>MC-3R</td>
<td>F: 5’-CGGTGGCCGACATGCTGTAAGTG-3’ B: 5’-TGAGGAGCATCATGGCGAAGAACA-3’</td>
<td>366</td>
<td>15,17</td>
</tr>
<tr>
<td>MC4/NM005912</td>
<td>MC-4R</td>
<td>F: 5’-CAATAGCACAAGACAAAGACT-3’ B: 5’-GACAAACAGAGCGCAAACAG-3’</td>
<td>566</td>
<td>15,17</td>
</tr>
<tr>
<td>MC5/NM005913†</td>
<td>MC-5R</td>
<td>F: 5’-CATTGCTGTGGAGGTGTTCT-3’ B: 5’-GCCTCATGTATGTGGTAG-3’</td>
<td>357</td>
<td>15-17</td>
</tr>
</tbody>
</table>

*Sequences as deposited in the National Center for Biotechnology Information (NCBI) database under the given accession number. †Size denotes the number of base pairs of the amplicon. ‡mRNA sequence for MC-2R is a co-linear but truncated form of the MC-5R.
Table II. alpha-MSH does not suppress collagen synthesis at the transcriptional level in HDF*

<table>
<thead>
<tr>
<th>Gene product</th>
<th>N/A</th>
<th>M</th>
<th>T</th>
<th>T + M</th>
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</thead>
<tbody>
<tr>
<td>Col alpha1(I)</td>
<td>22.2±1.1</td>
<td>22.9±2.7</td>
<td>55.6±9.6</td>
<td>55.5±2.3</td>
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<tr>
<td>Col alpha2(I)</td>
<td>81.6±9.6</td>
<td>95.2±14.4</td>
<td>149.2±20.3</td>
<td>135.2±23.9</td>
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<tr>
<td>Col alpha1(III)</td>
<td>28.8±2.8</td>
<td>28.6±3.8</td>
<td>76.2±14.8</td>
<td>52.3±3.8</td>
</tr>
</tbody>
</table>

*HDF were stimulated with 10−6 M alpha-MSH (M), 10 ng/ml TGF-beta (T) or both agents (T + M) for 12 hrs. †Relative mRNA levels were measured by real-time PCR and normalized for GAPDH. Data are means ± SEM from 3 independent experiments. ‡P<0.05 versus untreated cells (N/A).
Table III. Effect of alpha-MSH on number of immunoreactive interfollicular dermal cells in mice treated with TGF-beta,

<table>
<thead>
<tr>
<th>Marker</th>
<th>PBS/BSA</th>
<th>T</th>
<th>T + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>12.4±6.8†</td>
<td>39.3±3.2†</td>
<td>13.7±4.4†</td>
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<tr>
<td>Alpha-SMA</td>
<td>10.4±3.9†</td>
<td>30.0±9.1†</td>
<td>17.1±6.5†</td>
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</table>

*Mice were injected intracutaneously on the neck with 800 ng TGF-beta, (T) or 25 µg alpha-MSH (M) plus TGF-beta, (T+M), or PBS/BSA for 3 consecutive days. Number of immunoreactive cells was determined in 3x3 viewing areas of deparaffinized skin sections stained for vimentin and alpha-SMA as outlined in Material and Methods. Data are means ± SD from 3 independent experiments. †p<0.001, ‡p<0.02.
### A

<table>
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<tr>
<th></th>
<th>Cell lysates</th>
<th>Supernatants</th>
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<tbody>
<tr>
<td></td>
<td>N/A</td>
<td>T</td>
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<tr>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

α₁(III)

α₁(V)

α₂(V)

α₁(I)

α₂(I)

### B

![Bar chart showing PICP (ng/ml) for different conditions: N/A, M, T, T+M.](Fig. 1)
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Collagen metabolism—a novel target of the neuropeptide alpha-melanocyte-stimulating hormone
Markus Böhm, Michael Raghunath, Cord Sunderkotter, Meinhard Schiller, Sonja Ständer, Thomas Brzoska, Thomas Cauvet, Helgi B. Schiöth, Thomas Schwarz and Thomas Luger

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