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ORIGINAL PAPER

α-Melanocyte-Stimulating Hormone Gene Transfer Attenuates Inflammation after Bile Duct Ligation in the Rat

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Abstract Cholestasis occurs in a wide variety of human liver diseases, and hepatocellular injury is an invariant feature of cholestasis causing liver dysfunction and inflammation, promoting fibrogenesis, and ultimately leading to liver failure. α-Melanocyte-stimulating hormone (α-MSH) is a potent anti-inflammatory agent in many models of inflammation, suggesting that it inhibits a critical step common to different forms of inflammation. The aim of this study was to investigate whether the gene transfer of α-MSH could attenuate hepatic inflammation after bile duct ligation in the rat. Studies were performed in bile duct-ligated (BDL) rats. Hydrodynamic-based gene transfection with α-MSH plasmid via rapid tail vein injection was performed 30 min after ligation of bile duct. The endpoints were studied as markers of inflammation 7 days after bile duct ligation. α-MSH expression in liver via a single administration of naked plasmid was demonstrated. Liver inflammation index, including neutrophil infiltration and serum alanine aminotransferase, were significantly reduced in α-MSH gene transfer rats. Markers for liver inflammation, including expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and inducible NO synthase (iNOS) mRNA, as assessed by real-time PCR, were also attenuated by α-MSH gene therapy. Expression of iNOS protein in liver diminished after α-MSH gene transfer. Consistent with these data, hepatic stellate cells (HSC) and Kupffer cells were markedly inhibited in α-MSH gene-treated rats. Our findings show that gene transfer of α-MSH could attenuate hepatic inflammation after bile duct ligation in the rat.

Keywords α-Melanocyte-stimulating hormone · Bile duct ligation · Cholestasis · Gene transfer · Inflammation

Introduction

Cholestasis occurs in a wide variety of human liver diseases [1]. Although the pathogenic events culminating in cholestasis differ in each disease, hepatocellular injury is an invariant feature of cholestasis, causing liver dysfunction and inflammation, promoting fibrogenesis, and ultimately leading to liver failure. Retention and accumulation of toxic hydrophobic bile salts within hepatocytes are, in part, responsible for hepatocellular inflammation during cholestasis [2, 3]. Until a definitive cure for cholestasis becomes available, any new treatment that allows for the rescue of both functional and molecular levels of inflammation would be a significant advance.

The α-melanocyte-stimulating hormone (α-MSH) is a 13-amino-acid-long neuropeptide produced by intracellular cleavage of the proopiomelanocortin hormone. The

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anti-inflammatory activity of α-MSH has been demonstrated in various disease models including arthritis, septic shock induced by hepatic injury, and endotoxemia/ischemia, suggesting that α-MSH is a promising candidate therapeutic drug for inflammatory diseases [4–7]. The anti-inflammatory effects of α-MSH involve a reduction in the expression of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ, and interleukin-1, -6, and -8 (IL), and thus inhibit the inflammatory actions of leukocytes [7, 8]. In addition, over-production of a vasoactive mediator, nitric oxide (NO) by inducible NO synthase (iNOS) plays an important role in the pathophysiology of inflammation. Furthermore, hepatic stellate cells (HSC), resident perisinusoidal cells of the liver, are normally quiescent fat-storing cells, but undergo activation to a myofibroblast-like phenotype with expression of α-smooth muscle actin (α-SMA) during liver injury [9]. HSC activation is important in liver fibrosis formation after liver injury. Little was known about the mechanism of α-MSH gene transfer in liver after cholestasis. Could these inflammatory events of cholestasis be controlled by α-MSH transfection?

We have developed direct administration of a plasmid vector containing the genes of interest through venous injection [10–12]. This approach represents a promising new strategy to deliver and express foreign genes in vivo and has obvious advantages including the easy preparation of a large amount of plasmids, continuous expression of targeted protein, and its proven safety in an animal model [10–12]. In this study, we describe a hydrodynamic-based, in vivo transfection procedure utilizing intravenous administration of naked α-MSH plasmid that results in significantly high levels of exogenous α-MSH protein expression in liver. To delineate the nature and mechanism of the α-MSH gene therapy in cholestasis, we evaluated the effects of intravenous α-MSH plasmid infusion on the bile duct-ligated (BDL) rat. The specific aims were to answer the following questions:

1. Does liver inflammation attenuate in the α-MSH plasmid-treated rats compared with vector controls during cholestasis?
2. Do alterations in liver messenger (m)RNA expressions of TNF-α, IL-1β, and iNOS occur after α-MSH gene transfer?
3. Could cellular localization of iNOS protein be changed by α-MSH plasmid-treated group compared with vector controls?

The overall objective of this study was to determine the anti-inflammatory effects of α-MSH gene transfer in cholestasis.

Materials and methods

BDL rat

The use and the care of the animals for these studies were reviewed and approved by the Institutional Animal Care and Use Committee at Chang Gung Memorial Hospital. We used male Sprague–Dawley rats weighing 120–150 g that had been provided by the National Science Council. Under isoflurane inhalation anesthesia, the peritoneal cavity was opened and the common bile duct was double-ligated and cut between the ligatures (BDL rat). Controls underwent a sham operation that consisted of exposure but not ligation of the common bile duct (n = 15). Seven days after BDL, rats were killed by exsanguinations under deep isoflurane anesthesia using the following procedure. The peritoneal cavity was opened, and blood samples were taken from the infrahepatic vena cava, followed by immediate cannulation of the suprahepatic vena cava with a 20-gauge catheter; after the catheter was secured with 5-0 silk ligatures, the portal vein was cut. Using phosphate-buffered saline (PBS; pH 7.4) containing 137 mmol/l NaCl, 2.7 mmol/l KCl, 8 mmol/l $Na_2HPO_4 \cdot 7H_2O$, and 1.5 mmol/l KH_2PO_4, blood was flushed out of the liver via the suprahepatic vena cava catheter, to eliminate blood contamination in the tissue. The liver was cut into small pieces and used for the experiments described below.

Animal grouping, plasmid injection, and α-MSH protein expression

The recombinant α-MSH expression plasmid was a kind gift from Dr. Hedley (Zycos, Lexington, MA, USA) [13]. In brief, the fusion construct that encoded the 13 amino acids of α-MSH in-frame with the first domain of mouse serum albumin driven by a cytomegalovirus promoter (pCMV-MoLFa) was cloned. The construct that encoded only the first domain of mouse serum albumin (pCMV-SMo195) was used as a vehicle control. These plasmids were purified using an EndoFree Plasmid Giga Kit (Qiagen, Valencia, CA, USA). The rats were divided into α-MSH and vehicle groups, and plasmids of pCMV-MoLFa and pCMV-SMo195 were, respectively, injected into these rats (n = 15 in each group). Plasmid DNA was administered to rats by a hydrodynamic-based gene transfer technique via rapid injection of 10 ml DNA solution through the tail vein 30 min after BDL [10–12]. Briefly, a certain amount of plasmid DNA (400 μg) was diluted in 10 ml of saline and injected via the tail vein into the circulation within 5–10 s.

For analysis of α-MSH peptide expression, blood samples were obtained from cardiac puncture under isoflurane anesthesia 1, 3, and 7 days after BDL (n = 5 in each

group). The amount of plasma α-MSH was measured using an enzyme-linked immunosorbent assay (ELISA) method with minimum detectable concentration of 0.15 ng/ml according to the user's manual (Phoenix Pharmaceuticals, Belmont, CA, USA).

Histopathological and immunohistochemical examination

Seven days after BDL, livers were removed and fixed overnight in 10% buffered formalin ($n = 5$ in each group). Ten-micrometer sections were stained with hematoxylin-eosin for histological evaluation. Immunohistochemical staining was carried out using antibodies to α-MSH (1:100; Chemicon, Ijssel, Netherlands), iNOS (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-smooth muscle actin (α-SMA) (1:100; DakoCytomaton, Glostrup, Denmark), and ED2 monoclonal antibody (1:100; Serotec, Oxford, UK). Sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Positive expression was detected with diaminobenzidine (DAB; Sigma Chemical, St. Louis, MO, USA).

A negative control of immunohistochemical studies (α-MSH, iNOS, α-SMA, and ED2) was incubated as above without primary antibodies. Five random sections of each liver were examined. The number of cells with positive staining per high-power field (magnification x400) was quantitated respectively. All data are presented as means ± SEM of cell quantifications of five random sections.

Determination of serum total bilirubin and alanine aminotransferase activity

Serum alanine aminotransferase (ALT) and total bilirubin levels were measured using commercially available assay kits following the manufacturer's instructions (Sigma Diagnostics Kit no. 505 for ALT and 550 for bilirubin; Sigma Chemical).

Quantitative TaqMan RT-PCR

To confirm the expression levels of IL-1β, TNFα, and iNOS mRNA, we conducted quantitative RT-PCR. Total RNA was obtained from whole liver using the RNAqueous^{R} kit (Ambion, Austin City, TX, USA), according to the manufacturer's instructions ($n = 5$ in each group). To avoid genomic DNA contamination, which may interfere with PCR amplification, total RNA was treated with 0.2 U/μg TURBO DNase (Ambion) for 30 min at 37°C. The reverse transcriptase reaction was performed using 1 μg total RNA , oligo(dT)s, and the Advantage RT-for-PCR kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

The levels of mRNA were measured by the real-time quantitative PCR method using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For each treatment four distinct amplifications were carried out to amplify IL-1β cDNA, TNFα cDNA, iNOS cDNA, and GAPDH cDNA .The amplifications were performed in 50 μl volume containing 50 ng of cDNA, 25 μ of 2× TaqMan® Universal PCR Master Mix (Applied Biosystems), and 2.5 μl of 20× Assays-by-Design Buffer (Applied Biosystems). The sequences of the primers and TaqMan probes used in the study are summarized in Table 1 and the endogenous control gene GAPDH was designed by Applied Biosystems (Assay ID: Rn99999916_sl). The conditions for real-time PCR were preheating at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of shuttle heating at 95°C for 15 s and at 60°C for 1 min. IL-1β, TNFα, and iNOS mRNA levels from each treatment were normalized to the corresponding amount of GAPDH mRNA levels.

All samples were run in triplicate. For the detection of IL-1β mRNA after immunoprecipitation (IP) of messenger ribonucleoproteins (mRNPs), changes among sham, α-MSH, and vehicle groups at different IP conditions were based on the shift of threshold cycle (CT), the fractional cycle number at which the amplified target reaches a significant threshold. The higher the starting copy number of the target, the sooner a significant increase in signal was observed as a lower CT number. For detection of IL-1β mRNA in liver, quantitation of gene expression by real-time RT-PCR was evaluated using the Comparative CT Method as per the manufacturer's guidelines. The analysis of the relative quantitation required calculations based on the CT as follows:

1. ΔCT, the difference between the mean CT values of the samples evaluated with IL-1β-specific primers and those of the same samples evaluated with GAPDH-specific primers
2. ΔΔCT, the difference between the ΔCT values of the samples and the ΔCT value of the calibrator sample
3. $2^{-\Delta\Delta Ct}$, which yields the relative mRNA units representing the fold induction over the control

The same quantitation method was used to calculate TNFα and iNOS mRNA expression in liver.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) from at least three separate experiments. Differences between groups were compared using an analysis of variance (ANOVA) for repeated measures and a post hoc Dunnett t test to compare for multiple comparisons. P values less than 0.05 ($P < 0.05$) were considered significant.

Table 1 The sequences of the primers and TaqMan probe

Primer	Sequence
IL-1β forward primer	CTGCTAGTGTGTGATGTTCCCATTA
IL-1β reverse primer	TCAGACAGCACGAGGCATTTT
IL-1β TaqMan probe	CACTGCAGGCTTCG
TNFα forward primer	GGCTGCCCCGACTATGTG
TNFα reverse primer	CGGAGAGGAGGCTGACTTTC
TNFα TaqMan probe	CAGCCGATTTGCCACTTC
iNOS forward primer	AGGTGCACACAGGCTACTC
iNOS reverse primer	CCAGCTCTTTCTGCAGGATGT
iNOS TaqMan probe	CCCAAGGTCTACGTTCAAG

Note. IL-1β, interleukin 1β; TNFα, tumor necrosis factor α; iNOS, inducible NO synthase

Results

In vivo α-MSH expression in liver by a single administration of naked plasmid

The presence of α-MSH in serum was analyzed with an ELISA kit (minimum detectable concentration = 0.15 ng/ml). As shown in Fig. 1, a single administration of the pCMV-MoLFα plasmid resulted in marked expression of α-MSH in vivo. Levels of the α-MSH peptide in the circulation reached as high as 1.83 ± 0.30 ng/ml 1 day after gene transfer. The circulating level of α-MSH declined gradually; however, significant amounts of 0.93 ± 0.15 and 0.33 ± 0.15 ng/ml were still found 3 and 7 days after plasmid injection (Fig. 1). The levels of α-MSH in the vehicle and sham groups were all below the detection limit. Seven days after transfection, marked α-MSH staining was still shown in the gene transfer group (Fig. 2A), but not in the vehicle control group (Fig. 2B).

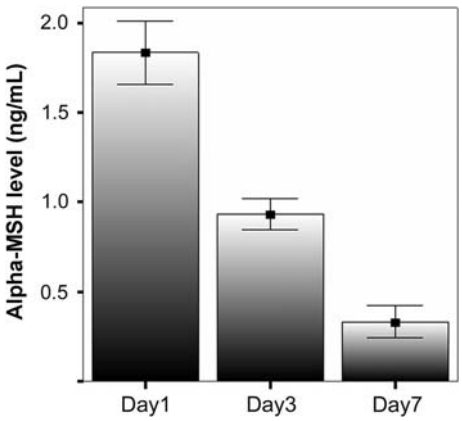


Fig. 1 The circulating α-melanocyte-stimulating hormone (α-MSH) levels in rats following a single injection of naked pCMV-MoLFα plasmid. Rats were rapidly injected via the tail vein with 10 ml of solution containing 400 μg of the plasmid. Levels of the α-MSH peptide in the circulation reached as high as 1.83 ± 0.30 ng/ml 1 day after plasmid injection. The significant amounts of 0.93 ± 0.15 and 0.33 ± 0.15 ng/ml were still found 3 and 7 days after plasmid injection. Levels at baseline and in the vehicle group were all below the detection limit (0.15 ng/ml; $n = 5$ in each group)

α-MSH gene transfer attenuated liver inflammation in BDL rats

In order to investigate whether liver inflammation attenuates in the α-MSH plasmid-treated rats during cholestasis, we checked the levels of ALT and total bilirubin 7 days after BDL. As shown in Fig. 3, a significant reduction in ALT levels was noted in the α-MSH group compared with the vehicle group 7 days (142.0 ± 37.7 vs. 209.5 ± 27.6 IU/l, $P = 0.004$) after BDL (Fig. 3A). Because of persistent cholestasis, no significant reduction in total bilirubin levels was noted in the α-MSH group compared with the vehicle group 7 days after BDL (9.35 ± 0.59 vs. 8.97 ± 0.61 mg/dl, $P = 0.849$; Fig. 3B).

The effects of α-MSH gene therapy on neutrophil infiltration in liver were measured using a quantitative method (Fig. 4). The number of neutrophils per high-power field (magnification ×400) was quantitated. Significant attenuation of neutrophil number was noted in α-MSH group, compared with the vehicle group (44.6 ± 14.8 vs. 252.6 ± 20.5 cells per high power field, $P < 0.001$) 7 days after BDL (Fig. 4C).

Effect of α-MSH gene transfer on α-SMA expression in BDL rats

The expression of α-SMA, a marker of activated HSCs [13], was detected by immunohistochemical staining 7 days after BDL (Fig. 5). The expression of α-SMA in the sham group was scanty (Fig. 5A). Intense specific staining for α-SMA was observed in the vehicle group (Fig. 5C). Significant inhibition of α-SMA expression was noted in the α-MSH group compared with the vehicle group (87.0 ± 30.0 vs. 423.6 ± 55.0 cells per high power field, $P < 0.001$, Fig. 5B, D). This indicated, BDL-induced α-SMA expression was attenuated by α-MSH gene transfer, so activated HSCs were effectively inhibited by α-MSH gene therapy in BDL rats.

α-MSH gene transfer attenuated TNFα, IL-1β, and iNOS mRNA expression in BDL rats

To determine whether α-MSH gene transfer alters liver cytokine or chemokine mRNA 7 days after BDL, we measured the changes in mRNA by quantitative RT-PCR (Fig. 6). As expected, the levels of TNFα mRNA of the vehicle group increased up to 4.58-fold compared with the sham group ($P = 0.006$), and α-MSH gene therapy

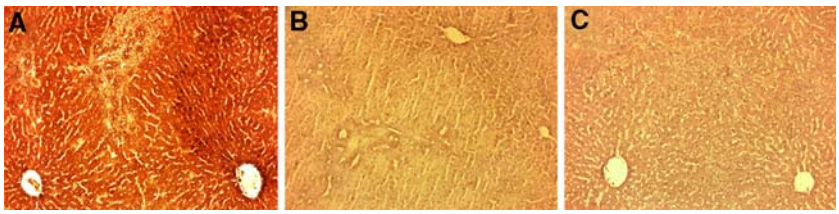
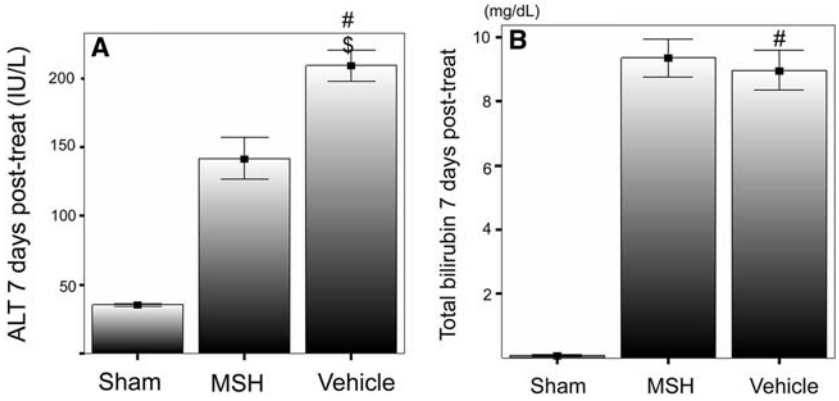


Fig. 2 α-MSH peptide expression in liver after gene transfer. **A** Immunohistochemical study of α-MSH in the gene transfer group showing marked staining 7 days after transfection. **B** No staining was shown in the vehicle group. **C** Negative control with omission of primary antibody in the same section of Fig. 2A (magnification, ×100)



Fig, 3 Liver function was preserved in α-MSH plasmid-treated bile duct-ligated (BDL) rats. **A** Serum ALT levels 7 days after BDL showing significant differences not only between sham and vehicle groups, but also between the pCMV-MoLFa (MSH) and vehicle groups. **B** Serum total bilirubin levels 7 days after BDL did not show a significant difference between the MSH and vehicle groups. Data were expressed as means ± SEM. (sham versus vehicle: #$P < 0.001$; MSH versus vehicle: \$$P < 0.05$; $n = 5$ in each group)

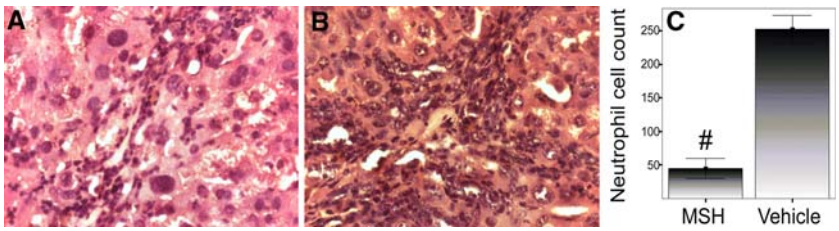


Fig. 4 Hematoxylin and eosin staining of liver histology 7 days after BDL shows attenuation of neutrophil infiltration in α-MSH-plasmid-treated BDL rats. **A** Small amount of neutrophils in the pCMV-MoLFa (MSH) group. **B** Large amount of neutrophils in the vehicle group. **C** Significant reduction in neutrophils in the MSH group. The number of positively stained cells per high-power field (magnification, ×400) was quantitated respectively. All data are presented as means ± SEM of five consecutive cell quantifications (MSH versus vehicle: #$P < 0.001$; $n = 5$ in each group)

could inhibit this up-regulation (0.52 ± 0.12 vs. 4.58 ± 1.60-fold, $P = 0.003$, Fig. 6A). The same situation was found in IL-1β mRNA expression. Compared with the sham group, 3.26-fold up-regulation of IL-1β mRNA was noted in vehicle group ($P = 0.001$), and α-MSH gene transfer was able to attenuate this up-regulation (0.94 ± 0.30- vs. 3.26 ± 0.68-fold; $P = 0.001$; Fig. 6B). Meanwhile, we also demonstrated an increase in iNOS mRNA up to 30.47-fold in the vehicle group, compared with the sham group ($P = 0.015$). Compared with the vehicle group, the α-MSH group showed significant down-regulation of iNOS mRNA expression (4.47 ± 2.89- vs. 30.47 ± 15.91-fold; $P = 0.025$; Fig. 6C).

Effect of α-MSH gene therapy on iNOS localization in BDL rats

We further investigated the localization of iNOS in the liver 7 days after BDL by immunohistochemical staining (Fig. 7). The protein expression of iNOS in the sham group was scanty (Fig. 7A). Intense staining for iNOS was observed in the pericentral area of the liver in the

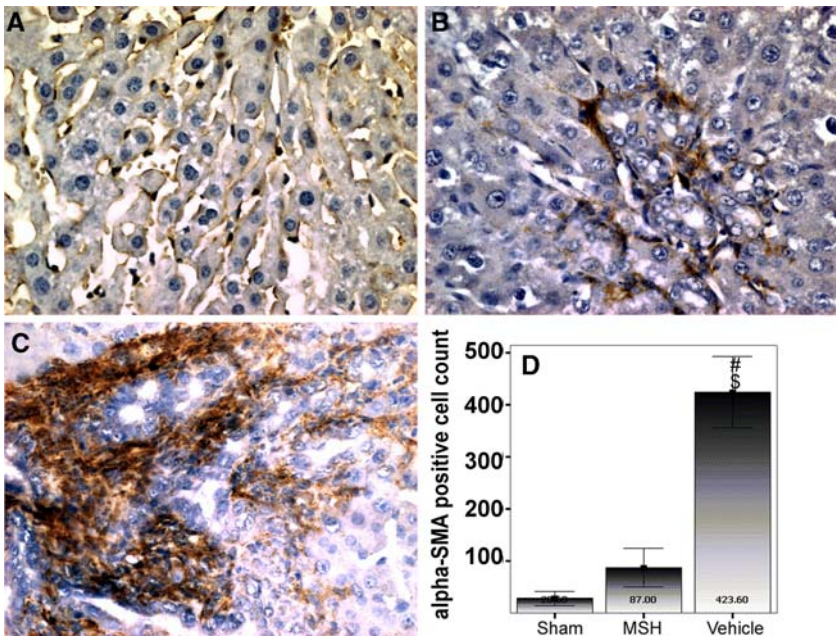


Fig. 5 Marker for hepatic stellate cell (HSC) activation increased in untreated compared with α-MSH-plasmid-treated BDL rats. **A** The expression of α-SMA in the sham group was scanty. **B** Marked inhibition of α-SMA expression was noted in the MSH group. **C** Intense specific staining for α-SMA was observed in the vehicle group. **D** Significant inhibition of α-SMA expression was noted in the MSH group compared with the vehicle group (sham versus vehicle: #$P < 0.001$; MSH versus vehicle: \$$P < 0.001$; $n = 5$ in each group; magnification, ×400)

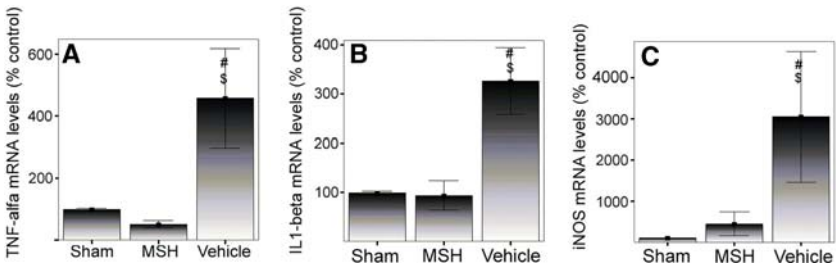


Fig. 6 α-MSH gene transfer attenuated tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and iNOS mRNA expressions in BDL rats using real-time PCR technology. **A** The levels of TNFα mRNA of the vehicle group significantly increased, compared with the sham group, and α-MSH gene therapy was able to inhibit this up-regulation. **B** Compared with the sham group, marked up-regulation of IL-1β mRNA was noted in the vehicle group, and α-MSH gene therapy was able to attenuate this up-regulation. **C** A significant increase in iNOS mRNA in the vehicle group compared with sham group was noted. Compared with the vehicle group, MSH group showed a significant down-regulation of iNOS mRNA expression (sham versus vehicle: #$P < 0.05$; MSH versus vehicle: \$$P < 0.05$; $n = 5$ in each group)

vehicle group (Fig. 7C), and α-MSH gene therapy could significantly inhibit iNOS localization in the liver (Fig. 7B, D).

α-MSH gene transfer attenuated Kupffer cells in liver

Finally, we investigated whether α-MSH gene transfer could attenuate Kupffer cells in BDL rats using ED2 staining (Fig. 8). In the sham group, there were very few Kupffer cells (Fig. 8A). Meanwhile, ED2-positive cells were easily demonstrated in the vehicle group (Fig. 8C), and Kupffer cells were hardly detected 7 days after α-MSH gene therapy (Fig. 8B). We demonstrated that α-MSH gene transfer could inhibit Kupffer cells during cholestasis. This finding also provides rationale of anti-inflammatory effect in α-MSH.

Discussion

Our study, using the BDL rat as a model of extrahepatic cholestasis, demonstrated a significant increase in hepatic inflammation and activation of HSCs 7 days after common BDL. Without proper treatment, hepatocellular injury is an invariant feature of cholestasis, causing liver dysfunction, promoting fibrogenesis, and ultimately leading to liver failure [3]. Our study was therefore designed to examine the effect and mechanism of α-MSH gene transfer after BDL.

To efficiently deliver exogenous α-MSH gene, we adapted an in vivo gene transfection procedure by rapid injection of a large volume of naked plasmid DNA solution via the tail vein [10–12]. It has been proposed that the injected DNA solution accumulates mainly in the liver

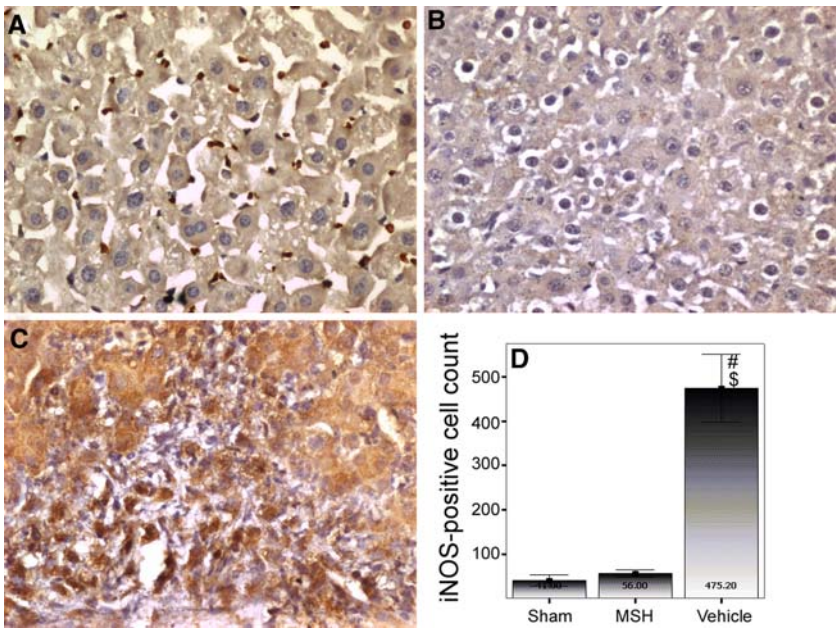


Fig. 7 α-MSH gene therapy reduced iNOS localization in BDL rats. **A** The protein expression of iNOS in the sham group was scanty. **B** Reduction in iNOS staining was noted in the α-MSH gene transfer group. **C** Intense staining for iNOS was observed in the pericentral area of the liver in the vehicle group. **D** α-MSH gene therapy was able to significantly inhibit iNOS localization in the liver (sham versus vehicle: #$P < 0.001$; MSH versus vehicle: \$$P < 0.001$; $n = 5$ in each group; magnification, ×400)

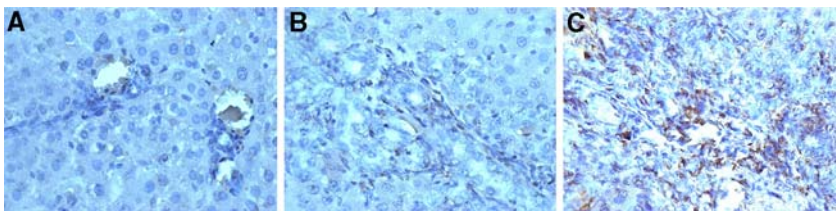


Fig. 8 α-MSH gene therapy attenuated Kupffer cells in liver using ED2 staining. **A** The expression of ED2 in the sham group was scanty. **B** Marked inhibition of ED2 expression was noted in the MSH group. **C** Intense specific staining for ED2 was observed in the vehicle group (magnification, ×200)

because of its flexible structure, which can accommodate a large volume of solution, and the hydrostatic pressure forces DNA into the liver cells before it is mixed with blood. Furthermore, breaking of the endothelial barrier by pressure has been proposed as the major mechanism responsible for the highly efficient expression in the liver [14]. Our data supported the fact that the transfected cells not only excrete α-MSH into the circulation (Fig. 1), but can also be directly modulated by the peptide inside them (Fig. 2).

The anti-inflammatory activity of α-MSH has been demonstrated in various disease models, including arthritis, septic shock induced by hepatic injury, and endotoxemia/ischemia, suggesting that α-MSH is a promising candidate therapeutic drug for inflammatory diseases [4–7]. However, the inherent instability and short half life of the peptide do not make it suitable for use as a therapeutic agent in humans [15]. From Fig. 1, we can see the efficient expression of α-MSH peptide in serum (1.83–0.33 ng/ml) for at least 7 days after injection of the gene, compared with the normal range of 0.01–0.03 ng/ml [16]. No wonder we were able to detect α-MSH peptide in neither the sham and nor the vehicle groups by ELISA with a minimum detectable concentration of 0.15 ng/ml. Within 7 days of injection of α-MSH gene, there was 10- to 180-fold circulating α-MSH peptide at any time in BDL rats. This is one of the pharmacokinetic reasons why a single injection of α-MSH gene can protect the liver during cholestasis. The other direct evidence of a high level of α-MSH peptide expression in the liver was demonstrated as long as 7 days after gene transfer.

To test the efficacy of α-MSH gene therapy in BDL rats, we first monitored liver functions such as ALT levels. Our data showed significant preservation of liver functions in the α-MSH plasmid transfer group at 7 days of BDL. We investigated the liver specimen 7 days after BDL, and found marked inhibition of neutrophil infiltration in the α-MSH gene therapy group. The role of neutrophils in liver injury following BDL has recently been reported highlighting a potential relationship between apoptosis and inflammation [17]. Furthermore, we found that Kupffer cells could be inhibited in α-MSH plasmid transfer rats compared with vector controls after BDL.

Moreover, we demonstrated HSC activation by α-SMA staining 7 days after BDL. Again, α-MSH gene therapy was able to attenuate HSC activation. Thus, our data suggested that cholestasis results in liver inflammation and HSC activation, while α-MSH gene therapy was able to attenuate these events.

Furthermore, we investigated the expression of cytokines such as TNF-α, IL-1β, and iNOS in mRNA levels using quantitative TaqMan RT-PCR. We found that α-MSH gene transfer significantly inhibits the increase in TNF-α mRNA expression 7 days after BDL. TNF-α is an important mediator in the pathogenesis of inflammation, and it stimulates neutrophil infiltration by alteration of adhesion molecules [18]. α-MSH interferes with the TNF-α pathway at many locations: it inhibits TNF-α mRNA induction in the liver, it inhibits TNF-α production in human peripheral blood monocytes, and prevents neutrophil accumulation [19]. Therefore, α-MSH inhibits both the synthesis and action of TNF-α. Meanwhile, IL-1β is also an important cytokine in inflammation. Here, we demonstrated that α-MSH gene transfer significantly inhibits the BDL-stimulated increase in IL-1β mRNA expression.

We further analyzed the mRNA expression of iNOS in BDL-stimulated hepatic tissues. Nitric oxide (NO) is a short-lived free radical that plays an important role in liver inflammation [20]. The formation of NO from L-arginine and molecular oxygen is catalyzed by the enzyme, nitric oxide synthase (NOS). Whereas the neuronal and endothelial isoforms of NOS are constitutively expressed, inducible NOS (iNOS) is significantly expressed after BDL. α-MSH gene transfer was able to attenuate up-regulation of iNOS in both mRNA and protein levels. Therefore, α-MSH gene therapy was able to effectively attenuate the up-regulation of TNF-α, IL-1β, and iNOS mRNA after cholestasis.

In summary, our findings suggest that during cholestasis in the rat, α-MSH gene transfer might be one of the therapeutic options for liver inflammation, until there is a definitive cure for obstructive jaundice.

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References

1. Trauner M, Meier PJ, Boyer JL (1998) Molecular pathogenesis of cholestasis. N Engl J Med 339:1217–1227
2. Greim H, Trulzsch D, Czygan P et al (1972) Mechanism of cholestasis. 6. Bile acids in human livers with or without biliary obstruction. Gastroenterology 63:846–850
3. Miyoshi H, Rust C, Roberts PJ et al (1999) Hepatocyte apoptosis after bile duct ligation in the mouse involves Fas. Gastroenterology 117:669–677
4. Chiao H, Foster S, Thomas R et al (1996) α-melanocyte-stimulating hormone reduces endotoxin-induced liver inflammation. J Clin Invest 97:2038–2044
5. Rajora N, Boccoli G, Catania A, Lipton JM (1997) Alpha-MSH modulates experimental inflammatory bowel disease. Peptides 18:381–385
6. Airaghi L, Lettino M, Manfredi MG et al (1995) Endogenous cytokine antagonists during myocardial ischemia and thrombolytic therapy. Am Heart J 130:204–211
7. Cannon JG, Tatro JB, Reichlin S, Dinarello CA (1986) Alpha melanocyte stimulating hormone inhibits immunostimulatory and inflammatory actions of interleukin 1. J Immunol 137:2232–2236
8. Rajora N, Boccoli G, Burns D et al (1997) Alpha-MSH modulates local and circulating tumor necrosis factor-alpha in experimental brain inflammation. J Neurosci 17:2181–2186
9. Friedman SL (2000) Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem 275:2247–2250
10. Wang CH, Liang CL, Huang LT et al (2004) Single intravenous injection of naked plasmid DNA encoding erythropoietin provides neuroprotection in hypoxia-ischemia rats. Biochem Biophys Res Commun 314:1064–1071
11. Wang CH, Jawan B, Lee TH et al (2004) Single injection of naked plasmid encoding α-melanocyte stimulating hormone protects against thioacetamide-induced acute liver failure in mice. Biochem Biophys Res Commun 322:153–161
12. Hung KS, Tsai SH, Lee TC et al (2007) Gene transfer of insulin like growth factor-I providing neuroprotection after spinal cord injury in rats. J Neurosurg Spine 6:35–46
13. Etemad-Moghadam B, Chen H, Yin P et al (2002) Inhibition of NF-kappa B activity by plasmid expressed alpha-MSH peptide. J Neuroimmunol 125:23–29
14. Liu F, Song YK, Liu D (1999) Hydrodynamic-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 6:1258–1266
15. Rudman D, Hollins BM, Kutmer MH et al (1983) Three types of alpha-melanocyte-stimulating hormone: bioactivities and half-lives. Am J Physiol 245:E47–E54
16. Rothuizen J, Biewenga WJ, Mol JA (1995) Chronic glucocorticoid excess and impaired osmoregulation of vasopressin release in dogs with hepatic encephalopathy. Domest Anim Endocrinol 12:13–24
17. Gujral JS, Farhood A, Bajt ML, Jaeschke H (2003) Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. Hepatology 38:355–363
18. Essani NA, Fisher MA, Farhood A et al (1995) Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. Hepatology 21:1632–1639
19. Catania A, Monno R, Motta P et al (1995) Influences of the neuropeptide alfa-MSH on cytokine production by peripheral blood mononuclear cells stimulated with gp120. VIII Research Project on AIDS, Rome, p 145
20. Li J, Billiar TR (1999) Nitric oxide. IV. Determinants of nitric oxide protection and toxicity in liver. Am J Physiol 276:G1069–G1073