Original Research

Inhibition of cartilage damage by pro-opiomelanocortin prohormone overexpression in a rat model of osteoarthritis

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Abstract

Pro-opiomelanocortin (POMC) is a precursor of various neuropeptides. POMC-derived neuropeptides are potent inflammation inhibitors and immunosuppressants. Evidence that osteoarthritis (OA) is an inflammatory disease is accumulating. We assessed whether intra-articular gene delivery of POMC ameliorates experimentally induced OA in a rat model. OA was induced in Wistar rats by anterior cruciate ligament-transection (ACLT) in the knee of one hind limb. Adenoviral vector encoding human POMC (AdPOMC) was injected intra-articularly into the knee joints after ACLT. The transgene expression and the inflammatory responses were evaluated using immunoblotting, immunohistochemistry and enzyme-linked immunosorbent assay. The treated joints were assessed histologically for manifestations of the disease. Human POMC was expressed in the chondrocytes and synovial membrane after the intra-articular injection. *POMC* gene transfer reduced nuclear factor-κB activity and the levels of interleukin-1β in HTB-94 chondrosarcoma cells and Raw 264.7 macrophages; it also reduced microvessel density in the synovium. Histological examination showed that symptoms of OA in AdPOMC-treated rats were less severe than in rats treated with either empty adenoviral vector (AdNull) or normal saline. Intra-articular injection of adenoviral vectors expressing POMC significantly suppressed the progression and severity of OA, and reduced inflammatory responses and angiogenesis. *POMC* gene delivery may offer novel therapeutic approach for treating OA.

Keywords: osteoarthritis, gene therapy, pro-opiomelanocortin, anterior cruciate ligament

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Introduction

Pro-opiomelanocortin (POMC) is a 31-kDa prohormone expressed predominantly in the central nervous system and pituitary gland. Several neuropeptides, including adrenocorticotropin (ACTH), melanotropins (α-, β-, γ-melanocyte-stimulating hormone [MSH]), lipotropins and β-endorphin (β-EP), are generated through the post-translational process of POMC.[1,2] ACTH and α-MSH peptides bind to melanocortin receptors, whereas β-EP binds to opioid receptors. Five melanocortin receptors (MC-1 to MC-5) have been cloned.[3] Although POMC and its derivatives were originally thought to be expressed only in the hypothalamus and pituitary gland, it is now known that they are also expressed in extraneural tissues. These peptides have multiple functions, including pigmentation, inflammation, immunomodulation, energy homeostasis and memory.[2,4–6] In addition, POMC has antiangiogenic and anti-inflammatory activities.[7–9]

Some of the POMC neuropeptides, such as α-MSH and β-EP, are potent inhibitors of the inflammation caused by proinflammatory and inflammatory cytokines.[4,10] α-MSH also modulates the immune response by impairing the function of antigen-presenting cells and T-cells.[11] It is immunosuppressive in various inflammation-related diseases such as arthritis.[2] Higher concentrations of α-MSH in the synovial fluid of arthritic joints have been correlated with a lower level of inflammation.[12] α-MSH is antiarthritic because it inhibits tumor necrosis factor (TNF)-α-induced matrix metalloproteinase (MMP)-13 expression by inhibiting p38 kinase phosphorylation and the downstream

activation of nuclear factor-κB (NF-κB).[13] Accumulating evidence suggests that osteoarthritis (OA) is an inflammatory disease.[14] During the progression of OA, the recruited T-cells in the knees secrete various chemokines and cytokines. The chemotactic macrophages then infiltrate the synovium and cause local inflammation. Angiogenesis, the formation of new blood vessels, is closely allied with inflammation: they mutually affect each other in the osteoarthritic joint and contribute to cartilage loss, osteophyte formation and synovial inflammation, which cause changes in the articular cartilage.[15] Proteolytic degradation of the articular cartilage matrix is the other major hallmark of OA. Increased production of MMPs induced by proinflammatory cytokines is responsible for further destruction of cartilage.[16,17]

In vivo POMC gene delivery, using a gene gun, of locally generated β-EP to rats with muscle or bladder pain indicated that POMC was processed in peripheral tissue.[18,19] The expression of melanocortin receptor in articular chondrocytes *in vitro* and in articular cartilage *in situ* has also been reported.[20] This makes human articular chondrocytes a good target for POMC delivery. Based on these findings, POMC may be a promising therapeutic agent for the treatment of inflammatory and degenerative diseases such as OA. In the current study, we evaluated the therapeutic effect of a local injection of AdPOMC, an adenoviral vector carrying the human *POMC* gene, in an anterior cruciate ligament-transection (ACLT) model of OA in rats.

Materials and methods

Adenoviral vectors, cell lines and animal models

To generate AdPOMC, the human POMC sense complementary DNA was constructed into an adenoviral plasmid as previously described.[8] An empty adenoviral vector, AdNull, was used as a negative control.[21] The HTB-94 human chondrosarcoma cell line was cultured at 37°C in 90% Leibovitz's L-15 medium. The mouse Raw 264.7 cell line and human primary synovial fibroblasts were cultured at 37°C in Dulbecco's modified Eagle's medium. Both of the media were supplemented with 10% fetal bovine serum, 1% glutamine and 50 μg/mL of gentamicin. Male Wistar rats (7 weeks old) were obtained from the Laboratory Animal Center of the National Cheng Kung University, Taiwan. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the university. To induce experimental OA, each rat was anesthetized with tiletamine hydrochloride plus zolazepam hydrochloride (10 mg/kg) (Zoletil 50; Virbac, Carros, France) and then subjected to a modified surgical procedure as previously described.[21,22]

Immunoblotting

To test POMC expression, six rats were intra-articularly injected with either AdPOMC or AdNull (3×10^7 $TCID_{50}$ [50% tissue culture infective dose]) and killed 72 h later. Tissue from the surfaces of their femoral condyles and their tibias, as well as synovium, was removed. The tissue was dissected for further homogenization in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Pierce, Rockford, IL, USA). Knee homogenates were subjected to immunoblot analysis using antibodies against human POMC cross-reacting anti-ACTH antibody (1:10,000) (Sigma-Aldrich, St Louis, MO, USA). HTB-94 cells were infected with AdPOMC at multiplicities of infection (MOI) of 10 and 100 and incubated for 24 h. The cells were supplemented with fresh medium and then incubated for an additional 24 h. The protein extract was then isolated and subjected to immunoblot analysis using antibodies against NF-κB p65 antibody (93H1) (1:1000) (Cell Signaling, Danvers, MA, USA). β-Actin expression was the quantitative control.

Immunohistochemistry

Seventy-two hours after the virus injection, the cartilage and synovium were removed, fixed and embedded in paraffin. Serial sections (5-μm thick) were cut and incubated with POMC antibody (1:125) (Sigma-Aldrich) at 4°C overnight. After they had been sequentially incubated for two hours at room temperature with the appropriate secondary antibody (1:400) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) and with aminoethyl carbazole as the substrate chromogen (Invitrogen, Zymed Laboratories, Camarillo, CA, USA), the slides were counterstained with hematoxylin. To analyze the antiangiogenic effects of POMC, on postsurgery day 14, the rats were injected with virus (3×10^7 $TCID_{50}$) once per week for two consecutive weeks and then killed four weeks after surgery. Vessel formation was determined by counting factor-VIII-positive vessels (von Willebrand's factor; Dako, Carpinteria, CA, USA). After sequential incubation with the appropriate secondary antibody and aminoethyl carbazole as substrate chromogen, the slides were counterstained with hematoxylin. Eighteen synovial sections from six rats per group (3 sections per rat) were analyzed. The synovial membranes from the medial, lateral and cranial recessuses were collected. Areas containing the highest number of capillaries were identified by scanning the sections at ×100 magnification. After the fields of high microvessel density were determined, individual vessels were counted at ×400 magnification. Microvessel density was determined by averaging the number of microvessels in three areas of highest vessel density at ×400 magnification in each section. The orientation of each section was random. To test MMP-13 expression, serial sections of cartilage were stained with MMP-13 antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After they had been sequentially incubated with the appropriate secondary antibody (1:400) (Jackson ImmunoResearch Laboratories) for two hours at room temperature and aminoethyl carbazole as the substrate chromogen (Invitrogen), the slides were counterstained with hematoxylin.

NF-κB activity assay

The NF-κB activity in HTB-94 cells was investigated by transfecting them with pWPXL-NF-κBLuc.[23] The cells grown in 24-well plates were infected with AdPOMC at an MOI of 10 and 100 for 24 h. After subsequent

transfection, the cells with this NF-κB-driven luciferase vector using lipofectamine (Invitrogen) were subcultured and incubated for an additional 24 h. TNF-α (20 ng/mL) was then added for 12 h. The NF-κB-driven luciferase activity in cells was determined using a kit (Dual-Light; Promega, Madison, WI, USA) in a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany).

Enzyme-linked immunosorbent assay

The primary human synovial fibroblasts were infected with AdPOMC at MOIs of 10 and 100 and incubated for 48 h. The conditioned medium was collected from fibroblast-like synoviocytes and added to the Raw 264.7 cells at 80% confluence on 24-well (200 μL/well) plates with or without TNF-α (20 ng/mL). After eight hours, the protein extract was isolated and the concentration of interleukin-1β (IL-1β) in the homogenates was determined using enzyme-linked immunosorbent assay.[21]

Treating OA with the *POMC* gene

Two weeks after surgery, the rats were divided into four groups of six and injected intra-articularly in the ACLT knees with either POMC or AdNull (3×10^7 $TCID_{50}$) once per week for two consecutive weeks. Rats in the normal saline (NS) group were injected with 100 μL of normal saline using the same schedule as described above. The sham-operated group received no treatment. Ninety days after ACLT, the rats were killed for histological examination.

Histological assessments

The sections were stained with Safranin O/fast green and the histopathological changes in the cartilage were examined using Mankin's histological grading as previously described.[24] The synovium was stained with hematoxylin and eosin. Histological changes in the synovial surface and subsynovial tissue were evaluated and scored as previously described.[25] Briefly, the grading system assigned separate scores based on two categories: (1) a synovial lining layer with three subcategories: hyperplasia of synovial lining cells (0–3 points), hypertrophy of synovial lining layer (0–3 points) and infiltration of inflammatory cells (0–3 points); and (2) subsynovial tissue with three subcategories: proliferation of granulation tissue (0–3 points), vascularization (0–3 points) and infiltration of inflammatory cells (0–3 points). Total scores in each category were calculated; the maximum score was 18 points.

Statistical analysis

Data are means ± standard deviation (SD). The statistical difference in NF-κB activity, IL-1β expression and microvessel density was analyzed using Student's *t*-test. Significance was set at $P < 0.05$. JMP 8.0 (SAS Institute Inc, Cary, NC, USA) was used to analyze the differences in Table 1. A one-way analysis of variance and Tukey's honestly significant difference test were used to compare differences between individual groups.

Table 1 Comparison of the articular cartilage change after treatment

Group	Osteoarthritic score	Synovitis score
NS ($n = 6$)	5.80 ± 1.10	6.67 ± 1.74
AdNull ($n = 6$)	6.08 ± 1.71	6.17 ± 1.75
AdPOMC ($n = 6$)	3.80 ± 0.80*	2.66 ± 0.98†‡
Sham ($n = 6$)	2.33 ± 1.03	1.58 ± 1.06

Criteria for score are defined in the Materials and methods
Data are expressed as the mean ± SD and analyzed using one-way analysis of variance and Tukey's honestly significant difference tests
*$P < 0.05$ compared with the NS and AdNull groups; †$P < 0.01$ compared with the AdNull group; ‡$P < 0.001$ compared with the NS group

Results

POMC expression in the knee joints after an intra-articular injection

An immunoblotting assay detected significantly more POMC delivered by adenoviral vector in AdPOMC-treated rats (Figure 1a). The protein detected in the AdNull-treated rats represented the endogenous POMC in tissue. AdPOMC was expressed in most chondrocytes, including those adjacent to the osteochondral junction and throughout the synovium (Figures 1b and c).

AdPOMC inhibited inflammation

Because NF-κB is an important regulator of the proinflammatory signaling pathway, we investigated the effect of

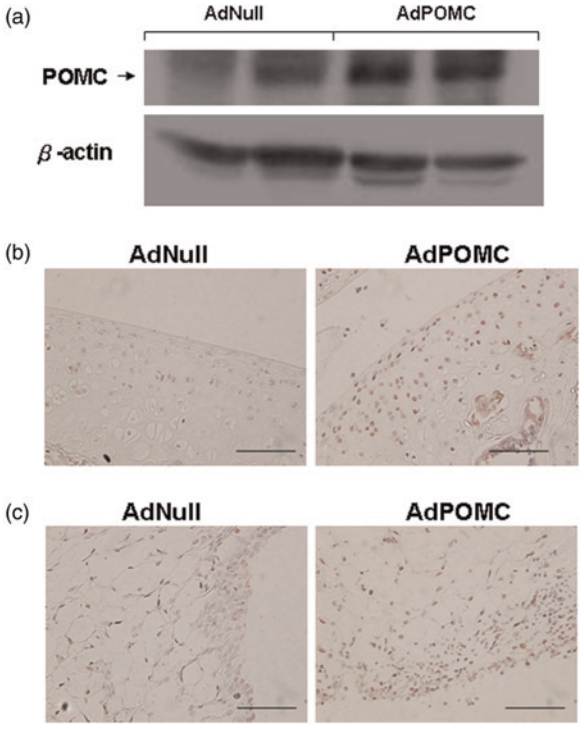

Figure 1 Expression of POMC in the knee joints of rats. (a) Immunoblot analysis showed that human POMC was detected in the joint extracts from AdPOMC-treated rats. β-Actin expression was the quantitative control. The expression of POMC was located in most chondrocytes (b) and synovium (c). Some endogenous POMC was also detected in the knees of the AdNull-treated rats (×200 magnification, scale bar = 100 μm). POMC, pro-opiomelanocortin (A color version of this figure is available in the online journal)

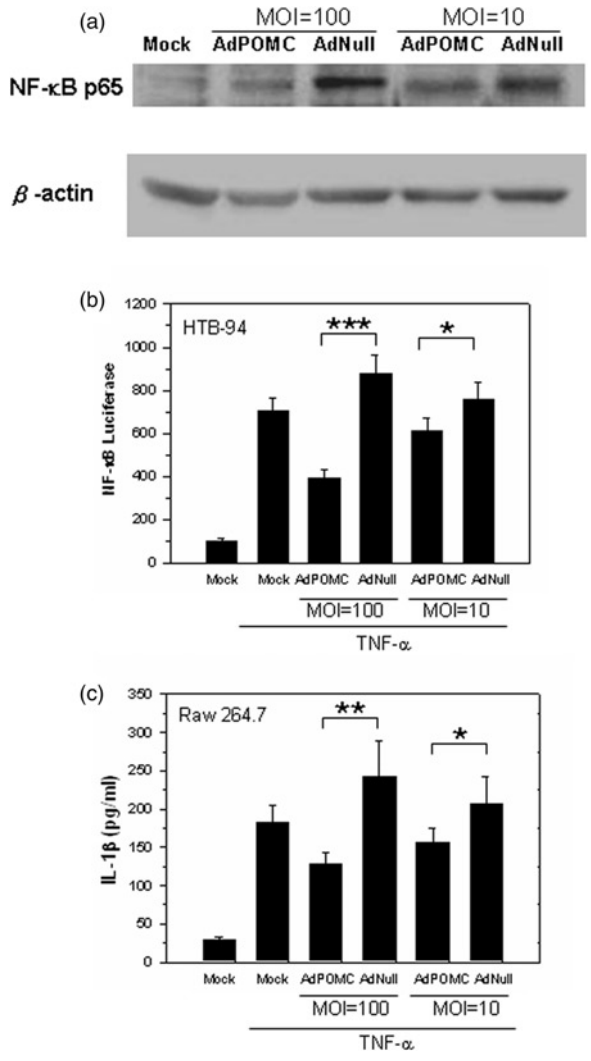

Figure 2 AdPOMC inhibited inflammation *in vitro*. (a) Immunoblot analysis showed that endogenous NF-κB expression was lower in AdPOMC-treated HTB-94 cells than in AdNull-treated cells. (b) In HTB-94 cells stimulated by TNF-α (20 ng/mL), NF-κB-driven luciferase activities were significantly lower in AdPOMC-treated than in AdNull-treated cells. (c) In Raw 264.7 cells stimulated by TNF-α, IL-1β expression was significantly lower in AdPOMC-treated than in AdNull-treated cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. POMC, pro-opiomelanocortin; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor; IL-1β, interleukin-1β

POMC gene delivery on the endogenous NF-κB activities in HTB-94 cells. The NF-κB levels were substantially lower in AdPOMC-treated cells than in AdNull-treated cells, especially at the higher virus titer (Figure 2a). The expression of TNF-α, a potent inflammatory cytokine in OA, can activate NF-κB; therefore, we next tested the effect of POMC on NF-κB activation after stimulation by TNF-α. NF-κB-driven luciferase activity in AdPOMC-infected cells was 44.6% of AdNull-infected cells at the MOI of 100% and 80.7% at the MOI of 10 (Figure 2b). In OA, the infiltrated monocytes in synovium will produce proinflammatory cytokines. To test the effect of POMC on cytokine production, IL-1β expression levels in Raw 264.7 cells after stimulation by TNF-α were examined. IL-1β expression was significantly lower in AdPOMC-treated cells than in AdNull-treated cells (Figure 2c). These results suggested that *POMC* gene delivery inhibited both endogenous NF-κB expression and TNF-α-induced proinflammatory factors *in vitro*.

Inhibition of angiogenesis by *POMC* gene delivery after ACLT

Fourteen days after ACLT, AdPOMC was intra-articularly injected into the knee joints of six rats (AdPOMC group) twice (days 14 and 21), and the microvessel density within the synovium was analyzed 28 d after surgery. Vessel density in the synovium was markedly higher in the ACLT and AdNull-treated groups (Figure 3a). The number of microvessels in the synovium was significantly lower (approximately 47%) in AdPOMC-treated (1.4 ± 0.54/HPF) (high power field) than in ACLT (2.6 ± 0.54/HPF, $P = 0.008$) rats, as well as between AdPOMC-treated and AdNull-treated rats (4.6 ± 1.14/HPF, $P = 0.0004$; Figure 3b).

Histopathological evaluation of AdPOMC-treated knee joints

We previously[26] showed that the most obvious changes in cartilage were on the medial femoral condyles. Therefore,

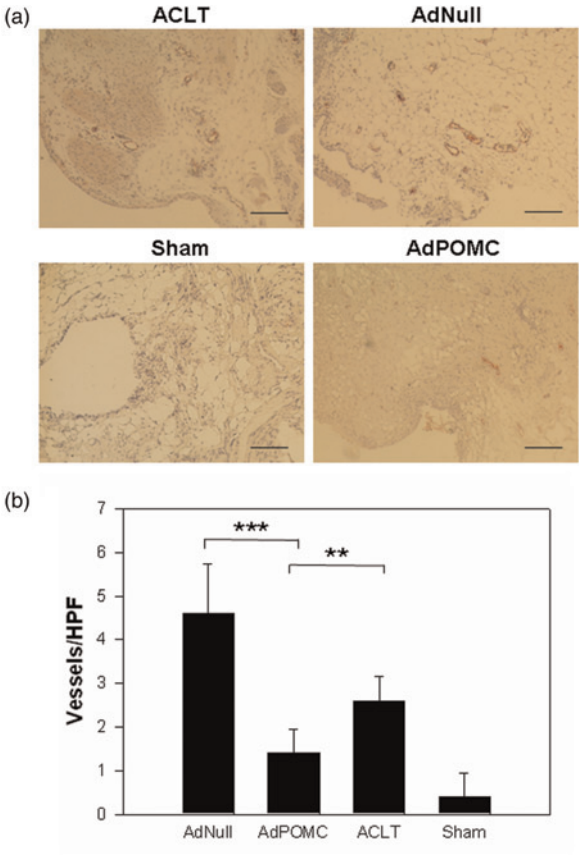

Figure 3 AdPOMC inhibited angiogenesis. (a) More blood vessels were distributed in the subsynovial tissues in AdNull-treated and ACLT rats than in AdPOMC-treated rats ($\times$200 magnification, scale bar = 100 μm). (b) Microvessel density was determined by averaging the number of vessels in three areas with the highest vessel count in each section ($\times$400 magnification). $^{**}P < 0.01$, $^{***}P < 0.001$. POMC, pro-opiomelanocortin; HPF, high power field; ACLT, anterior cruciate ligament-transection (A color version of this figure is available in the online journal)

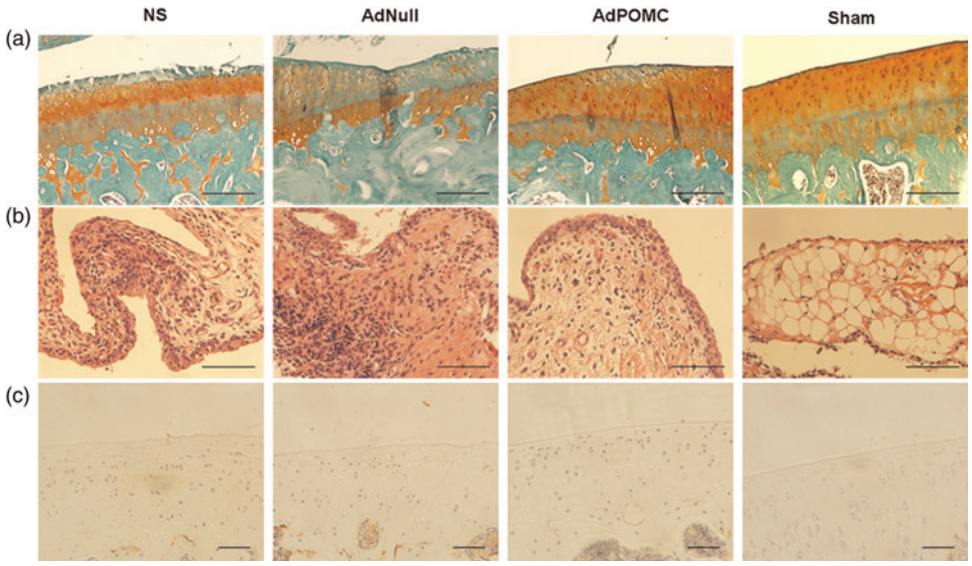

Figure 4 Evaluation of histological changes in the knee joints treated with AdPOMC. Cartilage on the medial femoral condyle is shown. (a) The NS specimen shows fibrillation and thinner surface cartilage than the AdPOMC and Sham specimens. The AdNull specimen shows chondrocyte loss and notch formation in the radial zone of cartilage (Safranin-O/fast green stain, ×200 magnification, scale bar = 200 μm). The AdPOMC specimen shows an irregular superficial layer of cartilage. The surface layer of cartilage in the Sham specimen was smooth and showed no significant changes. (b) The synovial membranes of the NS and AdNull specimens show hyperplasia and hypertrophy of synovial lining cells (H&E stain, ×400 magnification, scale bar = 100 μm). The AdPOMC specimen shows mild proliferation of synovial lining cells and mild cell infiltration. Synovial membrane from the sham-operated group showed no abnormal changes in the tissue. (c) Immunohistochemical staining shows lower MMP-13 expression in the medial femoral condyle from an AdPOMC-treated rat than from NS- and AdNull-treated rats (×200 magnification, scale bar = 100 um). POMC, pro-opiomelanocortin; NS, normal saline; MMP-13, matrix metalloproteinase 13 (A color version of this figure is available in the online journal)

the medial side of the femoral condyles was analyzed. Histopathological analysis of the saline-treated joint tissue showed a moderate fibrillation on the surface of cartilage (Figure 4a). The cartilage was not as thick, and there were fewer chondrocytes in the joints from the AdNull-treated group. In addition, the AdNull specimen shows notch formation in the radial zone of cartilage. Nevertheless, in the joints from the AdPOMC-treated group, the lesions were markedly less severe, and minimal irregularity was seen on the cartilage surface. In the sham-operated knee joints, the articular cartilage of the femur had a smooth surface. Furthermore, synovium in the NS- and AdNull-treated groups showed hyperplasia and hypertrophy of the lining cells and greater cellular infiltration (Figure 4b). In the AdPOMC-treated group, synovial abnormality was significantly lower than in the NS- and AdNull-treated groups; AdPOMC-treated rats showed slightly more cell proliferation in the synovial lining than did sham-operated rats. The osteoarthritic score in AdPOMC-treated joints was significantly ($P < 0.05$) lower than in NS- and AdNull-treated joints (Table 1). The synovitis score in AdPOMC-treated joints was also significantly lower than those in NS- ($P < 0.001$) and AdNull-treated joints ($P < 0.01$).

The inhibition of TNF-α can induce MMP-13 through POMC-derived peptides such as α-MSH. Because MMP-13 is a key mediator of cartilage degradation, we next examined MMP-13 expression in cartilage. Ninety days postsurgery, in NS- and AdNull-treated cartilage, MMP-13 expression was abundant; however, in AdPOMC-treated cartilage, it was almost totally inhibited (Figure 4c). Collectively, these results indicated that the *POMC* gene had modulated inflammation and angiogenesis in the knee joints of rats after ACLT and that it had attenuated the development of OA.

Discussion

We found that *POMC* gene delivery inhibited the activity of proinflammatory factor NF-κB and modulated the expression of IL-1β *in vitro*. Intra-articular injection of the *POMC* gene into rats inhibited inflammation and angiogenesis in ACLT-induced OA. POMC inhibited the expression of MMP-13, and attenuated the development of OA. A recent study[20] showed that human articular chondrocytes express POMC and its receptors – MC-1R, MC-2R and MC-5R – thereby modulate the biological functions of chondrocytes. Our results confirmed that POMC is a potential therapeutic for treating OA.

POMC encodes several neuropeptides and is expressed in both the central nervous system and extraneural tissue. Not only POMC and POMC-derived peptides but also corticotrophin-releasing and steroidal hormones are expressed in the skin.[27] The specific patterns of post-translational POMC processing dictate whether an individual cell releases certain downstream effectors. Because both POMC and melanocortin receptors are expressed in human chondrocytes,[20] POMC may have an anti-inflammatory effect on cartilage. Proinflammatory cytokines such as IL-1β and TNF-α released into the joint are responsible for the destruction of cartilage.[16,17] TNF-α-induced MMP-13 expression is regulated by NF-κB activation in human chondrocytes.[28] Our *in vitro* data showed that *POMC* gene delivery suppressed the expression of

endogenous NF-κB and TNF-α-induced NF-κB activity in chondrocytes. Different inflammatory pathways occurring in OA culminate with the activation of NF-κB, which is responsible for the expression of inflammation mediators such as TNF-α and IL-1β. Previous studies suggested that both human articular chondrocyte and chondrocyte cell line, CHON-002, were able to produce IL-1β through the activation of NF-κB signaling pathway.[29,30] In the Pin1-(a peptidyl prolyl isomerase) overexpressed HTB-94 cells, both of the NF-κB activity and the basal expression of IL-1β were increased.[31] Based on the previous and our studies, POMC could decrease the IL-1β production in HTB-94 cells. Our results showed that anti-inflammation was more prominent when a higher viral titer was used, which suggests that a higher concentration of POMC may compromise vector-induced inflammation. In OA, the infiltrated monocytes within synovium were the major source of IL-1β expression. Our data showed that the conditioned medium harvested from AdPOMC-infected fibroblast-like synoviocytes reduced IL-1β levels in macrophages, which suggested that a bystander effect may occur when AdPOMC is applied *in vivo*.

In addition to anti-inflammation, other mechanisms may participate in OA suppression by the *POMC* gene. Osteochondral angiogenesis causes cartilage loss, osteophyte formation and synovial inflammation, and facilitates the progression of OA.[32] *POMC* gene transfer may regulate the expression of an angiogenic factor, vascular endothelial growth factor, by inhibiting NF-κB activity. Other evidence[7] indicates that *POMC* gene delivery inhibited the migration and tube formation capability of endothelial cells. The derived neuropeptides ACTH and β-EP are also antiangiogenic.[33,34] We showed that, in addition to anti-inflammatory effects, *POMC* gene delivery appeared to inhibit OA disease progression through antiangiogenesis. Furthermore, in a rat model of chronic arthritic pain,[35] it inhibited the expression of β-EP in a brain region called the periaqueductal grey. This opioid peptide may alleviate OA-induced pain. In cartilage, the expression profile of neuropeptides derived from POMC remained unclear. However, it is possible that interactions among multiple neuropeptides derived from POMC *in situ* may be more therapeutic, which is why we used the *POMC* gene instead of a single gene from the processed peptides.

Our preliminary study showed that the acute immune response induced by surgery will gradually subside over two weeks (data not shown). In the surgically induced OA model, vascular invasion is one of the earliest events. The highest level of vascular invasion is detectable two weeks postsurgery and returns to control levels six weeks postsurgery.[36] To avoid the acute immune response caused by surgery, and achieve a better antiangiogenic effect, rats were injected intra-articularly in the ACLT knees with AdPOMC once per week for two consecutive weeks after two weeks postsurgery. Given the confined area where the virus is delivered, the vector-triggered inflammation for gene transfer in our model was limited. No significant inflammation was observed at the injection site 14 days after virus treatment.

Although local delivery of the *POMC* gene was effective for treating OA, several concerns should be considered before clinically applying *POMC* gene delivery. POMC expression may affect pigmentation, steroid synthesis, energy homeostasis and memory.[4–6] To avoid the possible adverse effects of POMC, particularly its interference with steroidogenesis and native immune functions, the optimal dose and regimen used must be carefully evaluated in additional studies.

In summary, our findings indicate that intra-articular *POMC* gene transfer inhibits cartilage destruction in ACLT knee joints. By targeting inflammation and angiogenesis, *POMC* gene transfer appears to have therapeutic potential for OA.

Author contributions: All authors participated in the design of the studies, analysis of the data and review of the manuscript. P-CS and A-LS made substantial contributions to the conception, design of the study and interpretation of data. J-LH and C-LW made substantial contributions to the acquisition, analysis of data and drafting the paper. I-MJ and M-HT conducted the experiment and edited the article. C-HL made substantial contributions to critical revision of the article for important intellectual content. H-YJ, P-RL and G-SL have made substantial contributions to provision of study materials and acquisition of data. P-CS and A-LS contributed equally to this work.

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