Adenovirus-Mediated Pro-opiomelanocortin Prohormone Gene Transfer Inhibited Cartilage Damage in Rat Knee Osteoarthritis

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Background: Pro-opiomelanocortin (POMC) is a precursor of various neuropeptides. The POMC-derived neuropeptides are potent inflammation inhibitors and immunosuppressant. The objective of this study was to assess whether intraarticular administration of POMC ameliorate experimentally induced osteoarthritis (OA) in a rat model.

Methods: OA was induced in Wistar rats by ACLT () in the knee of one hindlimb. Adenoviral vector encoding human POMC (AdPOMC) was injected intraarticularly into the knee joints after ACLT. The transgene expression and the inflammatory responses were determined by immunoblot analysis, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). The treated joints were assessed morphologically, radiographically, and histologically for disease manifestations.

Results: The expression of human POMC after intraarticular injection was identified in the chondrocytes and synovial membrane. POMC gene transfer reduced the NF-κB activity and the levels of interleukin-1 β (IL-1 β) in a human chondrocyte cell line and it also reduced microvessel density in synovium. Examination of gross morphology revealed that rats treated with AdPOMC had reduced severity of OA compared with the rats treated with either empty adenoviral vector (AdNull) or normal saline.

Conclusion: Local administration of adenoviral vectors encoding POMC significantly suppressed OA progression, accompanied by reduction of inflammatory response and angiogenesis.

Clinical Relevance: More evidences are accumulated to suggest that OA is an inflammatory disease. As an inflammatory inhibitor, POMC gene delivery may provide a therapeutic alternative for the treatment of OA.

Running Title: PRO-OPIOMELANOCORTIN PROHORMONE INHIBITED JOINT DAMAGE IN OA

Introduction

Pro-opiomelanocortin (POMC) is a 31-kDa prohormone expressed predominantly in the central nerve system and pituitary gland. Several neuropeptides, including adrenocorticotrophin (ACTH), melanotropins [α-, β-, γ-melanocyte-stimulating hormone (MSH)], lipotropins, and β–endorphin (β-EP) are generated through 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) had been cloned[3]. Although POMC and its derivatives were originally thought to be expressed in the hypothalamus and pituitary gland, it is now known that extraneural peptides also express POMC and POMC-derived peptides. These peptides possess multiple functions including pigmentation, inflammation, immunomodulation, energy homeostasis, and memory[2, 4-6]. In addition, POMC has been demonstrated to have anti-angiogenic effect and be able to suppress the tumor growth[7, 8].

Two of the POMC neuropeptides, such as α-MSH and β-EP are potent inhibitors of inflammation. Both of which can inhibit the production of pro-inflammatory and inflammatory cytokines[4, 9]. α-MSH can also modulate the immune response by impairing the function of antigen-presenting cells and T cells[10]. It has been reported to exert immunosuppressive function in various inflammation-related diseases including arthritis[2, 11]. Higher concentration of α-MSH observed in synovial fluid or the arthritis was correlated with the lower level of inflammation. The α-MSH could exert its anti-arthritis effect by inhibiting tumor necrosis factor-α (TNF-α)-induced matrix metalloproteinase 13 (MMP-13) expression through the inhibition of p38 kinase phosphorylation and the downstream activation of NF-κB[12]. Accumulating evidences suggest that Osteoarthritis (OA) is an inflammatory disease. During disease progression, the recruited T cells in knees secreted various chemokines and cytokines. The chemotaxis macrophages then infiltrated into synovium and

resulted in local inflammation. Angiogenesis, the formation of new blood vessels is closely interacted with inflammation. They mutually affect each other in the osteoarthritic joint and contribute to cartilage loss, osteophyte formation and synovial inflammation, and result in the articular cartilage changes[13]. Proteolytic degradation of the articular cartilage matrix is the other major hallmark of OA. Increased production of MMPs due to the stimulation by proinflammatory cytokines is responsible for further destruction of cartilage[14, 15].

Previous studies showed that *POMC* gene delivery in muscle or bladder using gene gun generated β–EP locally, indicating that POMC could be processed in the peripheral tissues[16, 17]. Recently, the expression of melanocortin receptor in articular chondrocytes *in vitro* and in articular cartilage *in situ* was reported[18]. 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 disease, such as OA. In the current study, we evaluate the therapeutic effect of AdPOMC, an adenoviral vector carrying human *POMC* gene, in the anterior cruciate ligament-transection (ACLT) model of OA in rats through local injection. Our results show that intraarticular delivery of the *POMC* gene attenuates the development of OA in rat ACLT knees.

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[19]. The HTB-94 human chondrosarcoma cell line The mouse Raw 264.7 cell line and human primary synovial fibroblast were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% glutamine, and 50 μg/ml gentamicin at 37℃. Male Wistar rats at the age of 7 weeks were obtained from the Laboratory Animal Center of the National Cheng Kung University. 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 National Cheng Kung University. To induce experimental OA, each rat was anesthetized with Zoletil 50 (10 mg/kg, Virbac, Carros, France) and then subjected to a modified surgical procedure as described previously[19, 20].

Immunoblotting

To test POMC expression, rats (n = 6) were intraarticularly injected with either AdPOMC or AdNull (3×10^7 $TCID_{50}$) and then killed 72 hours after the virus injection. After sacrifice, the tissue from the surface of femoral condyles and tibia as well as synovium was removed. All the tissues were dissected for further homogenization in phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Pierce, Rockford, IL). Knee homogenates were subjected to immunoblot analysis using antibodies against human POMC antibody (1:10000, Sigma-aldrich). HTB-94 human chondrosarcoma cells were infected with AdPOMC at an MOI of 10 and 100 and incubated for 24 hours. The cells were supplemented with fresh medium and incubated for additional 24 hours. The cell extract was then isolated and subjected to immunoblot analysis using antibodies against NF-κB antibody (1:1000, brand?). The expression of β-actin was used as 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, Saint Louis, MO) at 4℃ overnight. After they had been sequentially incubated with the appropriate secondary antibody (1:400; Jackson, city?) for 2 hours at room temperature and aminoethyl carbazole as the substrate chromogen (Invitrogen Zymed Laboratories, Camarillo, CA), the slides were counterstained with hematoxylin. To analyze the anti-angiogenic effects of POMC, on post-surgery day 14, the rats (n=6) were injected with virus (3×10^7 $TCID_{50}$) once per week for two consecutive weeks and then killed four weeks after surgery. Serial sections from synovium were stained with factor VIII (von Willebrand's factor; Dako, Carpinteria, CA). After sequential incubation with the appropriate secondary antibody and aminoethyl carbazole as substrate chromogen, the slides were counterstained with hematoxylin. To test MMP-13 expression, serial sections of cartilage were stained with MMP-13 antibody (1:100; Santa Cruz Biotechnology, Santa cruz, CA) at 4℃ overnight. After they had been sequentially incubated with the appropriate secondary antibody (1:400; Jackson) for 2 hours at room temperature and aminoethyl carbazole as the substrate chromogen (Invitrogen Zymed Laboratories), the slides were counterstained with hematoxylin.

NF-κB activity assay

The NF-κB activity in HTB-94 cells was investigated by luciferase activities assay. The cells grown in 24-well plates were infected with AdPOMC at an MOI of 100 for 24 hrs. After subsequent transfection the cells with NF-κB driven luciferase vector (Geneaid kit) using lipofectamine (Invitrogen, Carlsbad, CA) for 24 hrs, the cells were subcultured and incubated for additional 24 hrs. The TNF-α (10 ng/ml) were then added for 12 hrs. The NF-κB-driven luciferase activity in cells was determined using a Dual-Light kit (Promega, Madison, WI) in

a luminometer (brand?).

Enzyme-linked immunosorbent assay (ELISA)

The primary human synovial fibroblasts were infected with AdPOMC at an MOI of 10 and 100 and incubated for 48 hrs. The conditioned medium were harvested from fibroblasts and added to the Raw 264.7 cell at 80% confluence on 24-well (200 ul/well) in the presence or absence of TNF-α (10 ng/ml). After 12 hrs, the cell extract was isolated and the concentration of IL-1 β in the homogenates was determined by ELISA[19].

Treatment of osteoarthritis with *POMC* gene

Two weeks after surgery, the animals (n = 24) were divided into 4 groups. Rats were injected intraarticularly in the ACLT knees with either POMC or AdNull (3×10^7 $TCID_{50}$) once per week for 2 consecutive weeks. Rats in the normal saline (NS) group were injected with 100 ul of normal saline using the same schedule as described above. The sham-operated group received no treatment. Ninety days after ACLT, rats were sacrificed for histological examination.

Histologic assessments

The sections were stained with Safranin-O-fast green and the histopathologic change of cartilage was examined using the Mankin's histologic grading as described previously[21]. The synovium from ACLT was stained with hemotoxylin and eosin. The histological change of synovial surface and subsynovial tissue was evaluated and scored as previously described[22]. Briefly, the grading system assigned separated score based on two categories: synovial lining layer containing 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); subsynovial tissue containing 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, with a maximum of 18 points.

Statistical analysis

Data are means ± standard deviation (SD). The statistical difference in Figure 2 was analyzed using Student's *t* test. A value of $P < 0.05$ was regarded as statistically significant. JMP 5.0 (SAS Institute Inc., Cary, NC) was used to analyze the statistical differences in Table 1. Statistical significance between groups was estimated using one-way analysis of variance (ANOVA). To evaluate the differences between groups, we used Tukey's Honestly Significant Difference test set at 0.05. Statistical significance was set at $P < 0.05$.

Results

The expression of POMC in the knee joints after intraarticular injection

Immunoblotting assay revealed that substantial amount of human POMC delivered by adenoviral vector could be detected in AdPOMC-treated rats, compared to that from the control rats (Fig. 1A). The protein detected in NS-injected rats represented the endogenous POMC in tissues. The location of adenovirus-mediated POMC was expressed in most chondrocytes, including those adjacent to the osteochondral junction and throughout the synovium as determined by immunohistochemical staining (Figs. 1B, C).

Inhibition of inflammation by AdPOMC

Because NF-κB is an important regulator of proinflammatory signaling pathway, the effect of *POMC* gene delivery on NF-κB activities was investigated in HTB-94 cells. The NF-κB levels were significantly reduced after *POMC* gene delivery, especially at a higher virus titer (Fig. 2A). The NF-κB-driven luciferase activity in AdPOMC-infected cells was reduced to 45.84% of the AdNull-infected cells (Fig. 2B). (使用 TNF-apha 的用意要寫，不然 A 圖很突兀)The levels of IL-1β, induced in the presence of TNF-α, were reduced after AdPOMC infection (Fig. 2C). The results suggested that in addition to inhibiting the endogenous NF-κB, *POMC* gene delivery could also effectively repressed the TNFα-induced production of proinflammatory

factors *in vitro*.

Inhibition of angiogenesis by POMC gene delivery after ACLT

Fourteen days after ACLT, AdPOMC was intraarticularly injected into the knee joints twice (days 14, 21), and the microvessel density within the synovium was analyzed four weeks after surgery. There is an increase in the vessel density in the synovium undergoing ACLT and AdNull-treated groups (Fig. 3). However, two consecutive injections of AdPOMC could inhibit angiogenesis.

Histopathologic evaluation of AdPOMC-treated knee joints

Our previous studies showed the most obvious changes in cartilage were on the medial femoral condyles[23]. Therefore, the medial side of femoral condyles was analyzed. Histopathologic analysis of the saline-treated joint tissue showed a moderate fibrillation on the surface of cartilage (Fig. 4A). Joints from the AdNull-treated groups showed a decrease in cartilage thickness as well as fewer chondrocytes. Nevertheless, in the joints after AdPOMC treatment, the severity of lesion was remarkably reduced, as minimal irregularity was seen on the superficial of cartilage. In the sham-operated knee joints, the articular cartilage of the femur had a smooth surface. Furthermore, synovia in the NS- and AdNull-treated groups showed hyperplasia and hypertrophy of the lining cells and greater monocyte infiltration (Fig. 4B). In the AdPOMC-treated group, the abnormality in synovia was reduced. It

showed slightly more cell proliferation in the synovial lining than did sham-operated rats. The osteoarthritic score in joints treated with AdPOMC was significantly lower than in joints treated with AdNull and NS ($P<0.05$) (Table 1). The synovitis score in joints treated with AdPOMC was significantly lower than those in NS- and AdNull-treated joints ($P < 0.05$). Because MMP-13 is a key mediator of cartilage degradation, we next examined MMP-13 expression in cartilage. Ninety days post-surgery, in NS-and AdNull-treated cartilage, MMP-13 expression was abundant; however, in AdPOMC-treated cartilage, it was almost totally inhibited (Fig. 5). Collectively, these results indicated that the POMC gene modulated inflammation and angiogenesis in the knee joints of rats after ACLT and that it attenuated the development of osteoarthritis.

Discussion

The present study demonstrates that *POMC* gene delivery inhibits the activity of pro-inflammatory factor, NF-κB, and modulates the expression of IL-1β *in vitro*. Intraarticular injection of *POMC* gene into the rats reduces the inflammation and angiogenesis in the ACLT-induced OA. POMC could reduce the expression of MMP-13, and attenuates the development of OA. Recent finding showed that human articular chondrocyte could express POMC and its receptors, MC-1R, MC-2R and MC-5R, thereby modulate the biologic functions of chondrocytes[18]. Our results further confirmed that POMC could serve as potential therapeutics for the treatment of OA.

Our previous data showed that the acute immune response induced by surgery will subside gradually in two weeks (data not shown). In the surgically induced osteoarthritis model, vascular invasion is one of the earliest events. The highest level of vascular invasion is detectable two weeks post-surgery and returns to control levels six weeks post-surgery[24]. To avoid the acute immune response caused by surgery, and achieve better anti-angiogenic effect, rats were injected intraarticularly in the ACLT knees with AdPOMC once per week for 2 consecutive weeks two weeks post-surgery. 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.

Since the expression of both POMC and α-MSH were detected in human chondrocytes[18], they may be responsible for the anti-inflammatory in cartilage. In addition to α-MSH, POMC encodes several neuropeptides and produced in both central nervous system and extraneural tissues, the specific patterns of posttranslational POMC processing dictate whether an individual cell releases certain downstream effectors. In cartilage, the expressed profile of neuropeptides derived from POMC remained unclear and required for further investigation.

Our results suggested that in addition to anti-inflammation, other mechanisms may participate in OA suppression by *POMC* gene. Osteochondral angiogenesis could cause cartilage loss, osteophyte formation, and synovial inflammation and facilitate the progression of osteoarthritis[25]. *POMC* gene transfer may regulate the expression of an angiogenic factor, vascular endothelial growth factor, through the inhibition of NF-κB activity. Other evidence indicates that the *POMC* gene delivery could inhibit the migration and tube formation capability of endothelial cells[7]. Two neuropeptides, ACTH and β-EP had also been found to be responsible for the antiangiogenesis effect[26, 27]. Furthermore, in a rat model of chronic arthritic pain, the expression of β-EP was reported to be decreased in a particular brain region, called the periaqueductal grey. The production of this opioid peptide *in situ* may exert its

antinociceptive effect and alleviate the pain induced by OA. However, the possibility that interactions between multiple neuropeptides derived from POMC may also benefit for the therapeutic effect.

Although local delivery of POMC gene was effective for the treatment of OA, several concerns should be made before clinical application. The expression of POMC may affect pigmentation, steroid synthesis, energy homeostasis, and memory. To avoid possible adverse effects of POMC, particularly in the interference of steroidogenesis and native immune functions, the optimal dose and regimen used should be carefully evaluated and warranted for further studies. In summary, our findings indicate that intraarticular POMC gene transfer inhibits cartilage destruction in ACLT knee joints. By targeting to the inflammation and angiogenesis, POMC gene transfer appears to have therapeutic potential for osteoarthritis.

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FIGURE LENGENDS

Fig. 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 but not in those from AdNull-treated rats. The expression of β-actin served as the quantitative control. The expression of POMC was located in most chondrocytes (B) and synovium (C). Some endogenous POMC were also detected in the knees of the controls. (× 200 magnification).

Fig. 2. Inhibition of inflammation by AdPOMC *in vitro*. (A) Immunoblot analysis showed that NF-κB expression was decreased in HTB-94 cells after AdPOMC infection. (B) The NFκB-driven luciferase activities was inhibited in the presence of TNF-α (10 ng/ml) in HTB-94 cells after AdPOMC (MOI=100) infection. (C) Levels of IL-1β were reduced in the presence of TNF-α (10 ng/ml) in Raw-264.7 cells after AdPOMC infection. *$P < 0.05$, ***, $P < 0.001$.

Fig. 3. Inhibition of angiogenesis by AdPOMC delivery. More blood vessels were distributed in the subsynovial tissues in AdNull-treated and ACLT rats than in AdPOMC-treated rats (× 200 magnification).

Fig. 4. Evaluation of histological changes in the knee joints treated with AdTSP-1. The cartilage on the medial femoral condyle is shown. (A) The specimen from the

saline-treated group showed fibrillation and a decrease in cartilage thickness on the surface of cartilage. In the AdNull-treated group, chondrocyte loss and the notch formation occurred till the radial zone of cartilage were noted (Safranin-O/fast green stain, × 200 magnification). In the AdPOMC treatment group, the irregularity of the superficial layer of cartilage was observed. In the sham-operated group, the surface of cartilage layer was smooth, with no significant change. (B) In the saline- and AdNull-treated groups, the synovial membrane showed hyperplasia and hypertrophy of synovial lining cells (H&E stain, × 400 magnification). In the AdPOMC treatment group, mild proliferation of synovial lining cells was observed. A mild mononuclear cell infiltration was also seen. Synovial membrane from the sham-operated group showed no abnormal change in the tissues. (Scale bar = 2 mm).

Fig. 5. Immunohistochemical staining revealed that the expression of MMP-13 in the medial femoral condyle was decreased from Ad POMC-treated animals as compared with the saline treated- and AdNull-treated ones (× 200 magnification).