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Suppression of early experimental osteoarthritis by *in vivo* delivery of the adenoviral vector-mediated NF-κBp65-specific siRNA

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Summary

Objective: This study was to use adenoviral vector-mediated nuclear factor-κBp65 (NF-κBp65)-specific siRNA (Ad-siRNA$^{NF\text{-}\kappa Bp65}$) to suppress the progression of early osteoarthritis (OA) in rat model, and therefore to explore a new gene therapy for OA.

Methods: Reverse transcription polymerase chain reaction was performed to confirm the silencing effect of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ in cultured rat chondrocytes. Transection of the medial collateral ligament plus partial medial meniscectomy was operated in the knee of rats to establish OA model. Histological analysis was made to assess the morphological change of cartilage and synovium, and enzyme-linked immunosorbent assay was made to measure the expression of cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in synovial fluid. The silencing effect of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ on NF-κBp65 in cartilage and synovium of knee was measured with Western blot and the activation of NF-κB was measured with electrophoretic mobility shift assays.

Results: Ad-siRNA$^{NF\text{-}\kappa Bp65}$ can inhibit the activation of NF-κB and the expression of NF-κBp65 in cartilage and synovium of the knee, restrain the induction of IL-1β and TNF-α in synovial fluid, alleviate the inflammation of synovium and reduce the degradation of cartilage in early phase of experimental OA.

Conclusions: Ad-siRNA$^{NF\text{-}\kappa Bp65}$ can suppress the progression of the early experimental OA which suggests that Ad-siRNA$^{NF\text{-}\kappa Bp65}$ has potential to be a useful preventive and therapeutic agent for OA.

Key words: siRNA, NF-κBp65, Adenoviral vector, IL-1β, TNF-α, Osteoarthritis.

Introduction

Osteoarthritis (OA) is a degenerative disease that affects a large population and results in significant morbidity and disability. The initiating events that result in the cartilage degradation, sclerosis of subchondral bone as well as a variable degree of synovial inflammation that are characteristic of OA are poorly understood. Current research attributes these changes to a complex network of biochemical factors that lead to a breakdown of the cartilage macromolecules[1]. And pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), locally produced by the inflamed synovium and chondrocyte, also likely contribute to these alterations[2,3]. Both IL-1β and TNF-α can elicit the activation and proliferation of cultured synovial cells, and directly alter normal cartilage and bone metabolism because each can induce collagenase expression in synovial cells, inhibit proteoglycan synthesis in articular chondrocytes, and stimulate bone resorption *in vivo*[4,5]. Furthermore, IL-1β and TNF-α can induce the expression of other pro-inflammatory cytokines such as IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF)[6], which play major roles in inflammatory responses *via* the activation of a variety of transcription factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), and CAAT/enhancer binding protein (C/EBP) family members[7–9]. One of the major transcriptional circuits implicated in inflammation is the NF-κB/inhibitor of NF-κB (IκB) pathway. NF-κB is rapidly activated by pro-inflammatory cytokines like IL-1β and TNF-α, and is involved in the regulation of a large set of inflammatory response genes, including various cytokines and chemokines, acute-phase proteins, cell adhesion proteins, and immunoglobulins[7].

In recent years, gene therapy targeted at cytokines offers a new hope to OA treatment, and IL-1β and TNF-α are considered to be the principal inflammatory cytokines[10] and are thus natural targets for therapy for OA. Therefore, the use of biological agents that block the activity of these cytokines is the current focus[11]. These agents include TNF-α soluble receptor (TNF-sR)[12], IL-1 receptor antagonists (IL-1Ra)[13,14], anti-inflammatory cytokines[15] and inhibitors of catabolic enzyme[16]. As there are many pro-inflammatory cytokines, oxidants and other factors exerting action in initiation and development of OA, it is hard to get complete therapeutic effects by blocking the activity of one or two cytokines. So we attempted to inhibit signal pathway of NF-κB.

NF-κBp65 is a key active subunit in NF-κB transcription in several cell types[17,18], and our previous study provided evidences that interference of the expression of NF-κBp65

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with specific siRNA could effectively inhibit the transcription activation of NF-κB and the expression of matrix metalloproteinases-9 (MMP-9), nitric oxide synthase-2 (NOS-2) and cyclooxygenase-2 (COX-2) in induced chondrocytes[19]. So we attempted to use siRNA to interfere the expression of NF-κBp65 in animal to explore a new therapeutic approach for OA. At present, several techniques have been employed to introduce siRNA into vertebrate cells and animal models, and plasmid constructs have successfully been used to generate intracellular siRNA[20–22]. To improve the efficiency of the transfer of the expression cassette, recombinant retrovirus, recombinant adeno-associated virus and adenovirus based vectors have been used to introduce the expression cassettes into the target cells and animal models[23–27]. In this study, we used adenoviral vector to transfer siRNA into the knee joints of OA model, whose medial collateral ligament and partial medial meniscus were excised, to explore the anti-arthritic effect of the NF-κBp65-specific siRNA (Ad-siRNA$^{NF\text{-}\kappa Bp65}$). And we concluded that adenovirus mediated NF-κBp65-specific siRNA could restrain the induction of IL-1β and TNF-α, alleviate the inflammation of synovium, and reduce the degradation of cartilage in OA model at early phase.

Materials and methods

DESIGN OF HAIRPIN siRNA TEMPLATE OLIGONUCLEOTIDE

The target sequence of NF-κBp65-specific siRNA is 5′-AAGAGCATCATGA AGAAGAGT-3′ according to our previous study[19]. Ambion's siRNA target design online tool was utilized to design sense and antisense DNA oligonucleotides that encoded a hairpin siRNA template for the gene of NF-kBp65; sense template: 5′-TCGAGGAGCA TCATGAAGAAGAGTTTCAAGAGAACTCTTCTTCATG ATG CTCTTA-3′, antisense template: 5′-CTAGTAAGAG CATCATGAA AAGAGTTCTC TTGAAACTCTTCTTCATG ATGCTCC-3′. Two template oligonucleotides were synthesized by AuGCT Biotechnology Co., Ltd. (AuGCT, Beijing, PR China), annealed in 50 μl DNA annealing solution (Ambion, Austin, TX) at 90°C for 3 min, then slowly cooled to 37°C and incubated at 37°C for 60 min, the annealed siRNA template insert could be ligated into shuttle vector.

ADENOVIRAL VECTOR CONSTRUCTION

The p*Silencer*™ adeno1.0-CMV System (Ambion) was employed to produce recombination adenovirus. The annealed siRNA template insert was ligated into shuttle vector 1.0-CMV, identified with restriction endonuclease *Xho* I and *Spe* I, and confirmed by DNA sequence analysis. The adenoviral *Lac Z* backbone plasmid and the shuttle vector containing siRNA template were linearized with *Pac* I and co-transfected into human embryonic kidney (HEK)-293 cells using a calcium phosphate method to produce recombinant adenovirus. After adenovirus was expanded in HEK-293 cells, viral particles were purified with BD Adeno-X™Virus Purification Kits (BD Biosciences, Mountain View, CA), viral titer was determined by optical density at 260 nm and standard plaque assay with β-gal staining, the rate of transfection in chondrocytes was evaluated with β-gal staining, the silencing effect of Ad-siRNA was tested in chondrocytes with reverse transcription polymerase chain reaction (RT-PCR), and the recombinant adenovirus was stored at −80°C until used in experiment. The empty recombinant replication-deficient adenovirus (Ad-lacZ) and the recombinant adenovirus that encoded a scrambled siRNA (Ad-siRNA^{scrambled}), which were constructed in parallel, were used as control vectors throughout the study. All endonucleases were purchased from New-England Biolabs (Ipswich, MA, UK).

CHONDROCYTES CULTURE AND GENE TRANSFECTION

Articular chondrocytes were isolated from femoral heads and knees of Sprague Dawley (S-D) male rats (200–250 g, Experimental Animal Center of Peking University Health Science Center, Beijing, PR China) under aseptic conditions. The primary chondrocytes were obtained by enzymatic dispersal of cartilage as previously described[28]. Cells were seeded into Costar 24-well plates (Costar, Corning, NY) at a final density of 1×10^5 cells/well in Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and cultured for 3 days under normal growth conditions. Experiments were performed with the first or second passage cells. The chondrocytes were infected with recombinant adenovirus at 0, 10, 50, 100 and 200 multiplicity of infection (MOI) for 48 h, fixed with 1% glutaraldehyde for 15 min at room temperature, rinsed twice with phosphate-buffered saline (PBS, pH 7.4), and reacted to 0.1 M sodium-phosphate buffer, pH 7.5, 10 mM X-gal solution for 6 h at 37°C. Efficiency of gene transduction was assessed from microscopic observation of the cells that stained blue. The chondrocytes were infected with recombinant adenovirus at 100 MOI for 72 h. Total mRNA was isolated to perform RT-PCR.

mRNA EXPRESSION ANALYSIS

Total mRNA was isolated from cultured chondrocytes with Trizol (Invitrogen, Carlbad, CA). Isolated total RNAs (2 μg) were reversely transcribed for 45 min at 54°C and then amplified with a commercial kit (Access RT-PCR system®, Promega, Madison, WI) in a volume of 25 μl, employing specific primers of rat NF-κBp65. PCR analysis was conducted in the following conditions: 45 s at 94°C to denature the double-stranded DNA, 45 s at 60°C to allow for the annealing of the primers and 45 s at 68°C for primers extension. Amplifications were done for 35 cycles and fragments of PCR were 243 bp. The specific primers employed were (forward) 5′-TCACCAAAGAC CCACCTCACCG-3′ and (reverse) 5′-GGACCGCATTCAAGTCATAGTCCC-3′. PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining, and DNA bands were visualized under ultraviolet (UV) light and photographed.

IN VIVO EXPERIMENTAL DESIGN

Ninety S-D male rats (250–300 g, Experimental Animal Center of Peking University Health Science Center) were used in this study, 87 rats had operation on both knees, transection of the medial collateral ligament plus partial medial meniscectomy. Three days after the surgery, rats were used for the study. Handling and care of the animals were in accordance with the policies of the Peking University Health Science Center and of the National Institute of Health.

Fifteen S-D rats, which were averagely divided into five groups, were utilized for determining the optimal therapeutic range for Ad-siRNA$^{NF\text{-}\kappa Bp65}$. Both knees' joint of rats in five groups were transduced with one of five doses of virus (0.0, 1.6×10^5, 1.6×10^6, 1.6×10^7, and 1.6×10^8 pfu/ml). The

joints were lavaged with 200 μl PBS through routine arthrocentesis prior to transduction and at 7, 14, 21, 28, 35 days after transduction. The collected synovial fluids in right joints were analyzed with enzyme-linked immunosorbent assay (ELISA) to determine concentration of TNF-α, and in left joints were counted the number of inflammatory cells by use of routine clinicopathologic methods.

Three non-operated S-D rats were killed after transduction in knees of Ad-lacZ with 1.6×10^8 pfu/ml for 3 days. The joints were dissociated, which the joint cavity was exposed, and then were fixed with 2% paraformaldehyde, 0.1% glutaraldehyde for 1 h at room temperature, washed three times with PBS and reacted to 0.1 M sodium-phosphate buffer, pH 7.5, 10 mM X-gal solution for 4 h at 37°C. Reaction was stopped by washing the sample with PBS. After the color development, samples were observed macroscopically and took pictures.

Seventy-two operated rats were divided into five groups. Rats in group 1 ($n = 12$) received no treatment, one arthritic control group (group 2) of 12 rats received 1.6×10^8 pfu suspensions of empty recombinant adenovirus into both knees, another arthritic control group (group 3) of 12 rats received 1.6×10^8 pfu suspensions of recombinant adenovirus with siRNA^{scrambled} into both knees. Rats in group 4 ($n = 12$) were orally given aspirin (250 mg/kg/day) twice daily at 12 h intervals. Rats in group 5 ($n = 12$) received 1.6×10^8 pfu suspensions of recombinant adenovirus with siRNA$^{NF\text{-}\kappa Bp65}$ into both knees. A naive control group (group 6) of 12 rats was neither induced with arthritis nor given any treatment.

At 7 and 14 days after surgery, the knees were lavaged with PBS, levels of IL-1β and TNF-α in lavage fluids were measured using cytokine ELISA kits. At 14 days, rats were sacrificed and the cartilage and synovium were dissected and analyzed for effects of siRNA with histological analysis. The protein of cartilage and synovium was extracted and examined the silencing effect with Western blot. The nuclear protein of cartilage was isolated to make electrophoretic mobility shift assays (EMSAs).

ELISA

To lavage rat knee joints, 200 μl of PBS was injected into the joint space through the patellar tendon. After manipulation of the joint, the needle was reinserted, the fluid was aspirated and stored at −80°C until analysis. The levels of IL-1β and TNF-α in lavaged fluid were measured with ELISA kits (Biosource, Camarillo, CA) according to the manufacture's instructions.

WESTERN BLOT ANALYSIS

Total proteins were extracted from the comminuted cartilage and synovium with Trizol (Invitrogen), and protein concentrations were determined using the BCA Protein Assay Kit (Pierce) and their assay protocol. The blots were immersed in 20 ml of blocking buffer [5% skimmed milk and 1%Tween-20 in Tris-buffered saline (TBST)] for 2 h at room temperature and subsequently incubated overnight at 4°C with the primary antibodies: polyclonal anti-NF-κBp65 (1/500) (Santa Cruz, CA), followed by a secondary IgG (1/10,000) conjugated to horseradish peroxidase, then were washed three times for 15 min each in 20 ml of TBST with gentle shaking. Visualization of the immunocomplexes was conducted with the Luminol reagent kit (Santa Cruz) according to the manufacturer's specifications. The blots imaged by autoradiography were quantified by densitometry.

NUCLEAR PROTEIN ISOLATION AND EMSA

EMSA was used to study the effect of siRNA on NF-κB activation. Nuclear proteins of cartilage were isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. The concentration of proteins in samples was measured by the bicinchonninic acid (BCA) method. The oligonucleotides for the NF-κB consensus sequence were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACT CCCCTGAAAGGGTCCG-5′ which were end-labeled with biotin (synthesized by Beijing AuGCT Biotechnology Co., Ltd.). Complementary oligonucleotides were annealed in sodium Tris-Hcl EDTA (STE) buffer by slow cooling to room temperature in boiling water. Nuclear extracts (5 μg) were added in 20 μl of binding reactions and incubated for 20 min at room temperature. EMSAs were performed according to the manufacture's protocol of LightShift® Chemiluminescent EMSA Kit (Pierce). To establish the specificity of the reaction, negative controls without cell extracts and competition assays with a 200-fold excess of unlabeled oligonucleotide were performed. In competition assays, the corresponding unlabeled probe was added to the reaction mixture 10 min before the addition of the labeled probe.

HISTOLOGICAL ANALYSIS

Histologic evaluation was performed on sagittal sections of cartilage from the lesion areas on medial femoral condyle. After dissection, specimens were fixed in 10% buffered formalin for 24 h followed by decalcification in a mixture of 10% formic acid and 2% buffered formalin for 7—14 days. The fixed, decalcified tissue was embedded in paraffin and processed using conventional dehydrating and clearing agents, and 5-μm sections were cut to stain with hematoxylin and eosin (H and E) and toluidine blue.

The severity of OA lesions was graded on a scale of 0—6 by three independent observers using a modification of the Mankin method[29], namely, grade 0, normal; grade 1, surface irregularities; grade 2, pannus and surface irregularities; grade 3, superficial cleft formation; grade 4, deep but localized clefts down to bone; grade 5, large surface defects down to bone; and grade 6, complete loss of cartilage on the load bearing surface. The scoring system was based on the most severe histologic changes in the multiple sections.

Representative specimens of synovial membrane were dissected from underlying tissues. The specimens were fixed, embedded, sectioned (5 μm) as above, and stained with H and E and examined by light microscopy. The samples were quantitatively classified according to synovium hypercellularity and the extent of synovitis[29]: grade 0, normal synovia and underlying tissues; grade 1, mild or focal synovitis limited to surface or mild fibrosis of subsynovial tissues without synovial involvement; grade 2, moderate synovitis with beginning fibrosis of subsynovial tissues; grade 3, severe synovitis with moderate fibrosis of underlying tissues. Three synovial membranes of specimens were examined for scoring and the higher score of each was retained. The average was calculated and considered as a unit for the whole knee.

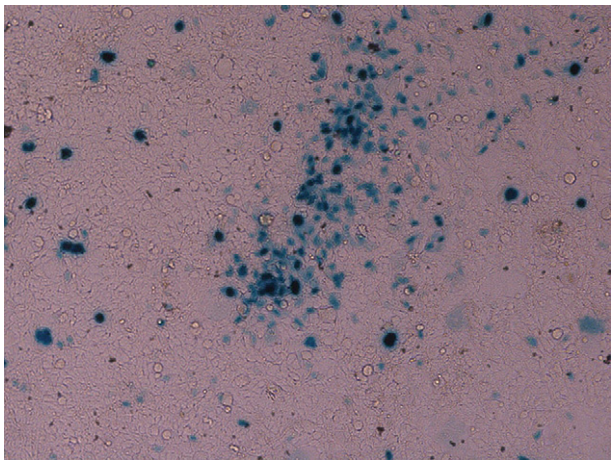

Fig. 1. The β-gal staining of adenovirus foci. After infection with recombination adenovirus 48 h, HEK-293 cells were stained with β-gal for 24 h and a comet-shaped adenovirus foci appeared (×400).

STATISTICAL ANALYSIS

All values, expressed as mean ± S.E.M., were subjected to Student's *t* test between two groups and one-way analysis of variance (ANOVA) among multiple groups employing the computer SPSS 10.0 statistic package, at a significance level of $P < 0.01$.

Results

THE DESIGN OF siRNA AND PRODUCTION OF RECOMBINANT ADENOVIRUS

Because there is gene of *Lac Z* in the adenovirus backbone plasmid, the β-gal staining was made to make out that the recombinant adenovirus was successfully packaged and infected into HEK-293 cells (Fig. 1). The viral titer was 8×10^8 pfu/ml, the rate of transfection of Ad-siRNA in chondrocytes at 0, 10, 50, 100 and 200 MOI was 0, 16.38 ± 1.98, 67.45 ± 7.63, 93.5 ± 8.56, and 98.21 ± 7.52, respectively. According to RT-PCR, we found that the expression of NF-κBp65 was evidently reduced (Fig. 2) and indicated that Ad-siRNA had silencing effect in chondrocytes.

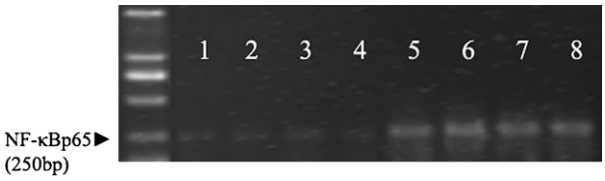

Fig. 2. The effect of Ad-siRNA on expression of NF-κBp65 in chondrocytes. The chondrocytes were infected with Ad-siRNA at 100 MOI for 72 h, total RNA was isolated and RT-PCR of NF-κBp65 was performed, the expression of NF-κBp65 was evidently decreased. Lanes 1, 2, 3, 4: infection with Ad-siRNA and lanes 5, 6, 7, 8: control.

IN VIVO DOSE TITRATION STUDY

After operation of knee, the expression of TNF-α in synovial fluid increased. And intra-articular injection of the Ad-siRNA$^{NF\text{-}\kappa Bp65}$ leads to dose-depended decrease in the concentration of TNF-α in synovial fluid aspirates, the lowest TNF-α concentration, which was the highest silencing effect of Ad-siRNA$^{NF\text{-}\kappa Bp65}$, occurred with 1.6×10^8 pfu/ml [Fig. 3(A)]. The marked, acute synovial fluid leukocytosis was not seen at any viral loads [Fig. 3(B)].

β-GAL STAINING OF JOINT

After injection of Ad-lacZ for 3 days, the joint was stained with β-gal, cartilage and synovium of joint were stained (Fig. 4). This indicated that adenovirus can infect into cartilage and synovium, and the gene of *lacZ* was expressed in chondrocytes and synoviocytes.

THE EFFECTS OF AD-siRNA$^{NF\text{-}\kappa Bp65}$ ON ACTIVATION OF NF-κB IN CARTILAGE

On EMSA (Fig. 5), the protein of NF-κBp65 in cartilage of OA had an obvious DNA binding (lane 4) in comparison with the protein in normal cartilage (lane 2). However, transduction of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ into cartilage showed a significant inhibition of NF-κB binding (lane 6), but Ad-siRNA^{scrambled} had no effect on NF-κB binding (lane 5). This binding reaction was specific since unlabeled NF-κB probe prevented the formation of the complexes with biotin-labeled NF-κB probe (lane 3).

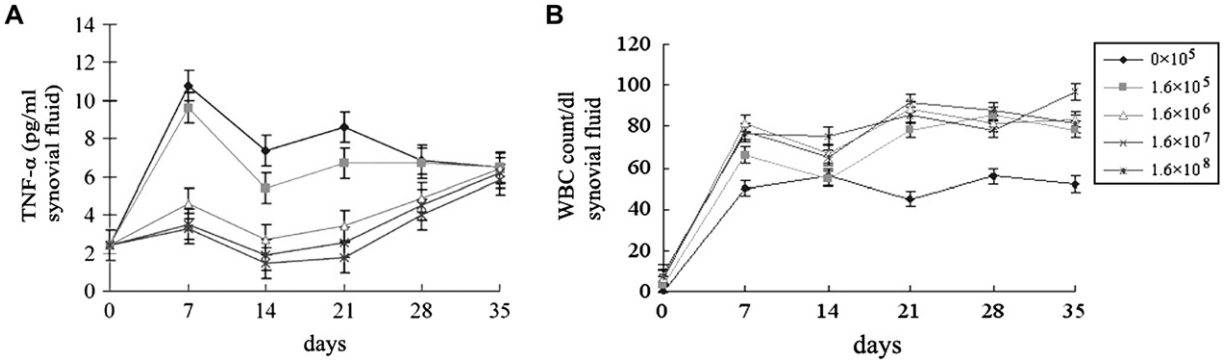

Fig. 3. Production of TNF-α *in vivo* following intra-articular injection of various amount of the Ad-siRNA$^{NF\text{-}\kappa Bp65}$: after induction of OA, the expression of TNF-α in synovial fluid increased. And intra-articular injection of the Ad-siRNA$^{NF\text{-}\kappa Bp65}$ leads to dose-depended decrease in the concentration of TNF-α in synovial fluid aspirates, the lowest TNF-α concentration occurred with 1.6×10^8 pfu/ml (A). The marked, acute synovial fluid leukocytosis was not seen at any viral loads (B).

Fig. 4. β-gal staining of joint: after injection of Ad-lacZ for 3 days, the joint was stained with β-gal, cartilage and synovium of joint were stained with blue.

THE EFFECTS OF AD-siRNA$^{NF\text{-}\kappa Bp65}$ ON EXPRESSION OF TNF-α AND IL-1β IN SYNOVIAL FLUID OF OA

To examine the effects of Ad-siRNA on the expression of TNF-α and IL-1β in synovial fluid, the knees of OA were lavaged and analyzed with ELISA. At 1 week, the expression of TNF-α and IL-1β in synovial fluid in group 1 (OA without treatment), group 2 (OA that injected with Ad-lacZ) and group 3 (OA that injected with Ad-siRNA^{scrambled}) was obviously increased compared with group 6 (normal), significantly decreased in group 4 (OA that treated with aspirin) and group 5 (OA that treated with Ad-siRNA$^{NF\text{-}\kappa Bp65}$) compared with group 3, and the expressions affected by

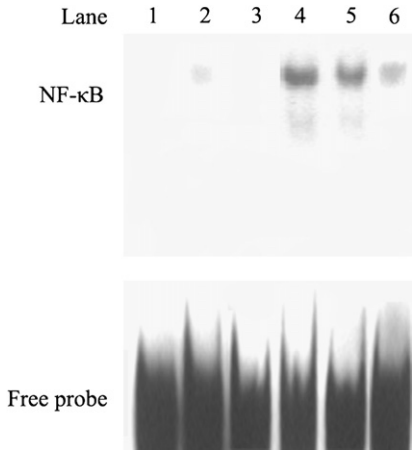

Fig. 5. The effect of Ad- siRNA$^{NF\text{-}\kappa Bp65}$ on NF-κB DNA binding in cartilage: The protein of NF-κBp65 in cartilage of OA had an obvious DNA binding (lane 4) in comparison with the protein in normal cartilage (lane 2). Ad-siRNA$^{NF\text{-}\kappa Bp65}$ could significantly inhibit the NF-κB binding (lane 6), but Ad-siRNA^{scrambled} had no effect on NF-κB binding (lane 5). This binding reaction was prevented with a 200-fold excess unlabeled NF-κB probe (lane 3).

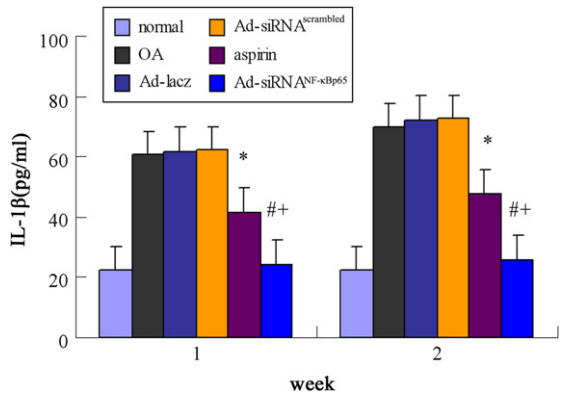

Fig. 6. The effects of Ad-siRNA and aspirin on the expression of IL-1β in the synovial fluid of OA at 1 and 2 weeks after surgery. $^{\#}P < 0.01$ vs OA without treatment, $^{*}P < 0.01$ vs OA without treatment, and $^{+}P < 0.01$ vs treatment with aspirin.

Ad-siRNA$^{NF\text{-}\kappa Bp65}$ was decreased greater than those affected by aspirin. At 2 weeks, the expression of TNF-α and IL-1β was similar to those of 1 week. It should be noted that the expression of TNF-α with Ad-siRNA$^{NF\text{-}\kappa Bp65}$ at 2 weeks was lower than the expression of group 6 (normal rats) (Figs. 6 and 7).

THE EXPRESSION OF NF-κBp65 IN CARTILAGE AND SYNOVIUM

Western blot revealed that the protein of NF-κBp65 in cartilage in group1 (OA without treatment), group 2 (OA that injected with Ad-lacZ) and group 3 (OA that injected with Ad-siRNA^{scrambled}) was significantly increased compared with group 6 (normal rats), and significantly decreased in group 5 (OA that treated with Ad-siRNA$^{NF\text{-}\kappa Bp65}$) compared with group 3 (Fig. 8), the expression of NF-κBp65 in synovium was similar to that of cartilage (Fig. 9), except that effect of Ad-lacZ and Ad-siRNA on synovial cells was greater than on chondrocytes. This indicated that Ad-siRNA could transfect into chondrocytes and synovial cells, and markedly inhibited the expression of NF-κBp65. This also showed that the effect of Ad-siRNA on synovial cells was more evident than on chondrocytes,

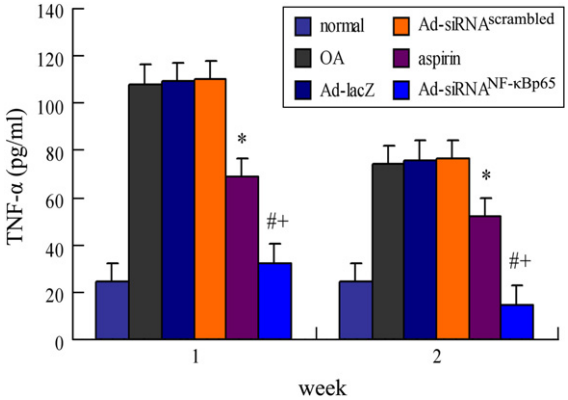

Fig. 7. The effects of Ad-siRNA and aspirin on the expression of TNF-α in the synovial fluid of OA at 1 and 2 weeks after surgery. $^{\#}P < 0.01$ vs OA without treatment, $^{*}P < 0.01$ vs OA without treatment, and $^{+}P < 0.01$ vs treatment with aspirin.

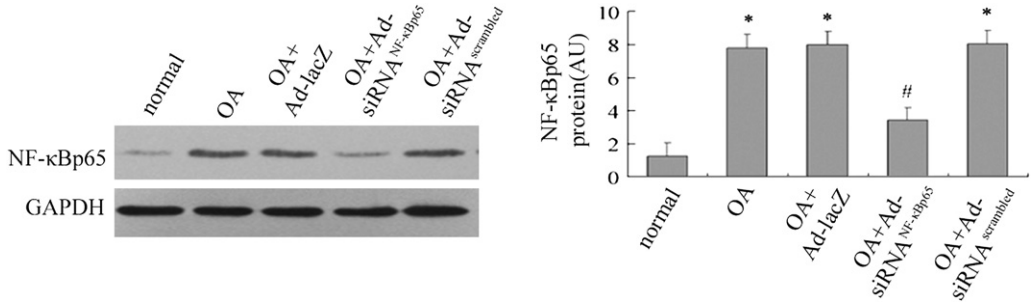

Fig. 8. The effects of Ad-siRNA on the expression of NF-κBp65 in cartilage. The cartilage was collected at 2 weeks after surgery, the expression of NF-κBp65 was assessed at the level of protein by Western blot. The densitometric quantification of NF-κBp65 was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as arbitrary units (AU). Representative Western blot analysis of NF-κBp65 and GAPDH is shown and graph shows the mean ± S.E.M. of the three independent experiments. $^*P < 0.01$ vs control.

and synovium was more susceptible to adenovirus than cartilage.

HISTOLOGIC EVALUATION OF ARTICULAR CARTILAGE

Specimens of group 1 at week 2 show a morphologic change characteristic at early stage of OA. The total histologic severity of lesions was similar in this group, transverse clefts occurred and resulted in loss of articular cartilage mass, fibrosis occurred at the surface of cleft degenerative cartilage, and several chondrocytes in one lacuna formed a cluster in intermediate layers. Toluidine blue staining for proteoglycan was distributed unevenly, the areas in the superficial and intermediate layers were mostly destained and only the areas around cell clusters were still darkly stained [Fig. 10(A) and (B)]. Group 2 and group 3 showed a similar degree of morphologic change with group 1 at week 2 [Fig. 10(C) and (D), (E) and (F)].

In group 4, chondrocyte cloning decreased, cartilage degeneration and proteoglycan loss and fibrosis alleviated compared with group 2 [Fig. 10(G) and (H)]. Group 5 showed a marked reduction in cartilage pathology, and degeneration and fibrosis of cartilage were slight. The loss of proteoglycan in the cartilage was observed by decreased staining with toluidine blue in the superficial and intermediate layers, and cell clustered in the intermediate layers [Fig. 10(I) and (J)]. As a control, normal cartilage sections from group 6 are shown [Fig. 10(K) and (L)]. The grade of severity of OA lesion in different groups was calculated, and there were significant difference between group 3, group 4 and group 5 (Fig. 11).

HISTOLOGY EVALUATION OF SYNOVIAL MEMBRANE

When compared with synovium from group 6 [Fig. 12(A)], sections from group 1, group 2 and group 3 showed a typical synovitis of OA [Fig. 12(B), (C) and (D), respectively], the synovium was dramatically thickened, highly fibrous, and hypercellular, with increased number of synoviocytes and infiltrating mononuclear leukocytes. The rats treated with aspirin had slightly smaller lesions on synovial sections than the rats in group 1 [Fig. 12(E)]. Joints treated with Ad-siRNA$^{NF\text{-}\kappa Bp65}$ showed a more slighter synovitis [Fig. 12(F)], synovial sections from this group of rats were more distinguishable from those from group 1 and group 3. The grade of severity of OA lesion in different groups was calculated, there were significant difference between group 3, group 4 and group 5 (Fig. 13).

Discussion

Although the etiology of OA is poorly understood, numerous animal models that mimic aspects of the disease have been developed to study the pathophysiology of the disease and to evaluate potential therapeutics. Besides a model of monosodium iodoacetate-induced OA[30], a variety of surgical models of OA in animals have been devised to accelerate the degenerative changes that occur over long periods of time in spontaneous OA. Some of the most commonly used models in larger animals are the Pondnuki model (anterior cruciate ligament (ACL) transection) in dog[31], lateral meniscectomy in sheep[32] and partial meniscectomy in rabbit[33]. Small animal models of surgical OA

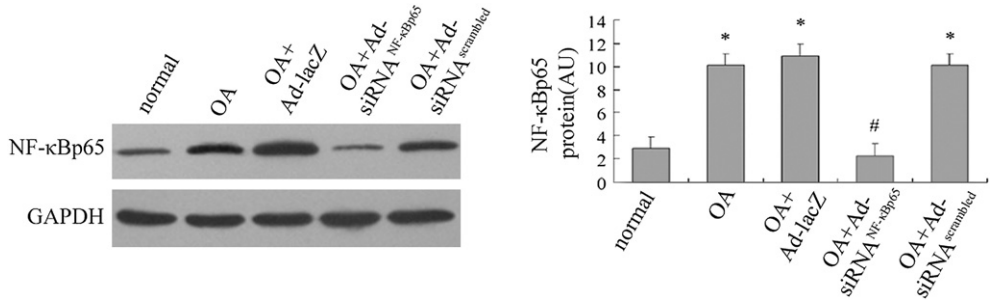

Fig. 9. The effects of Ad-siRNA on the expression of NF-κBp65 in synovium. The synovium was collected at 2 weeks after surgery, the expression of NF-κBp65 was assessed at the level of protein by Western blot. The densitometric quantification of NF-κBp65 was normalized to GAPDH and expressed as AU. Representative Western blot analysis of NF-κBp65 and GAPDH is shown and graph shows the mean ± S.E.M. of the three independent experiments. $^*P < 0.01$ vs control.

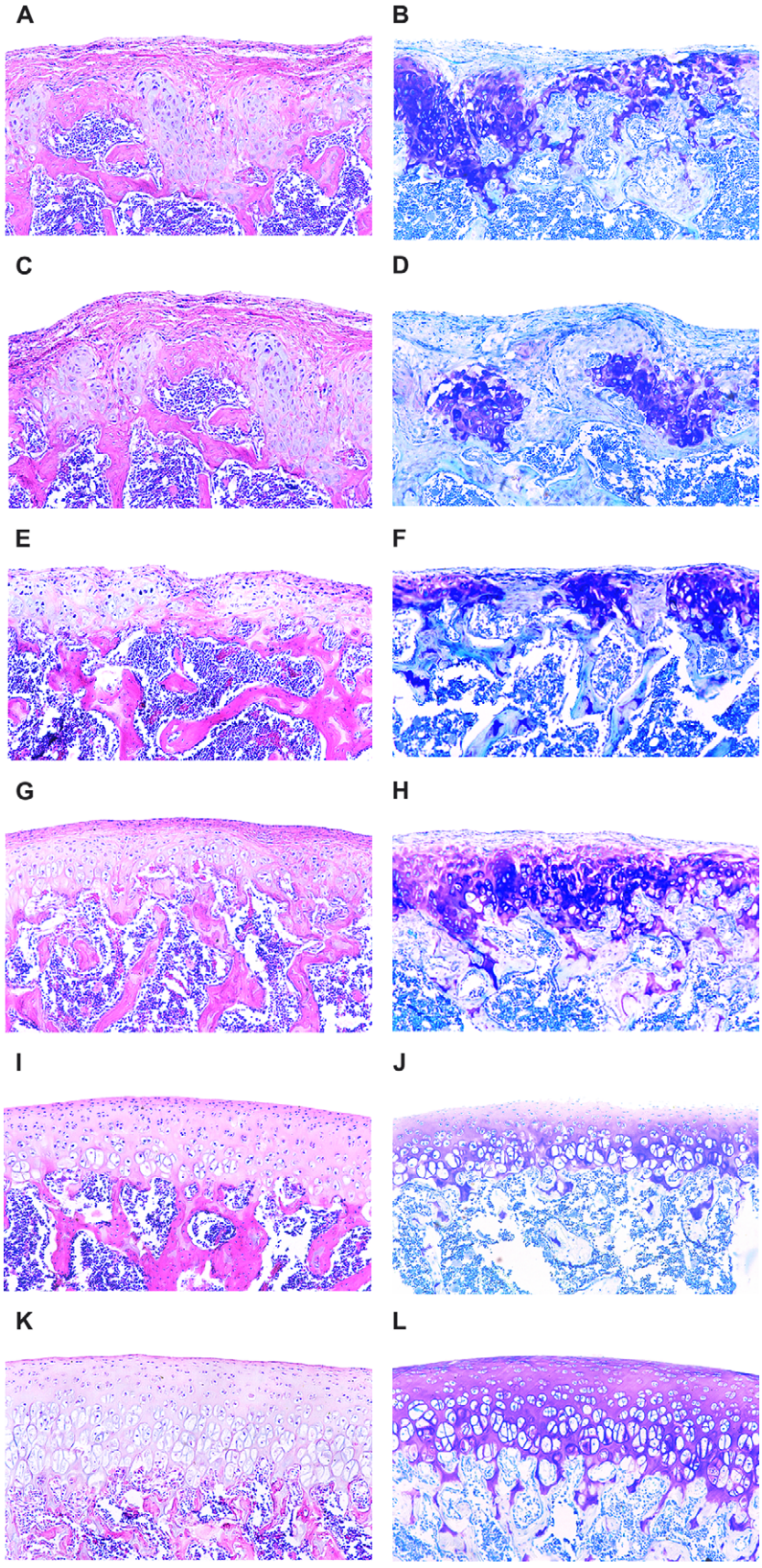

Fig. 10. Representative sections of articular cartilage from the medial femoral condyle of OA at 2 weeks after surgery were stained with H and E (left pane) and toluidine blue staining (right pane) of [(A) and (B)] OA without treatment, [(C) and (D)] OA that injected with Ad-lacZ, [(E) and (F)] OA that injected with Ad-siRNA^{scrambled}, [(G) and (H)] OA that treated with aspirin, [(I) and (J)] OA that received injection of Ad-siRNA$^{NF\text{-}\kappa Bp65}$, and [(K) and (L)] rats that neither induced with arthritis nor treatment ($\times$100).

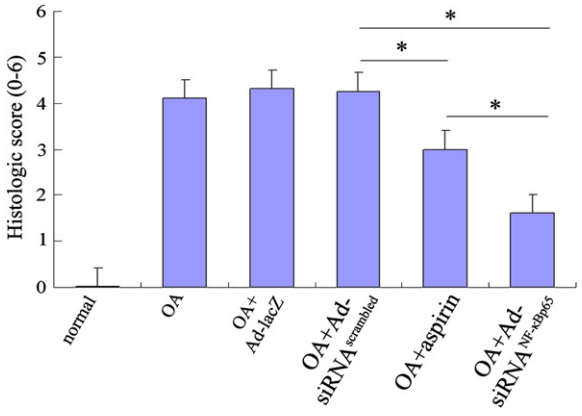

Fig. 11. Histologic grading of cartilage from femoral condyles of OA joints of rats at 2 weeks after surgery. Values are the mean ± S.E.M. total score (0–6 scale) of lesions from OA rats without treatment; OA rats with intra-articular injection of Ad-lacZ; OA rats with intra-articular injection of Ad-siRNA^{scrambled}; OA rats treated with aspirin; OA rats treated with intra-articular injection of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ and rats that neither induced with arthritis nor treatment. Asterisks (*) denote values which differ at $P < 0.01$.

have also been described including partial meniscectomy in guinea-pig[34], meniscus tear in the guinea-pig[35] and ACL transection in rat[36]. Janusz[37] induced OA in rats by surgical tear of the meniscus and found that moderate cartilage degeneration of the tibial plateau was observed at week 1 after operation with focal loss of chondrocytes and proteoglycan. The cartilage lesions increased slightly in depth at week 2 with some areas of focally severe cartilage degeneration. In our study, at week 2 after operation, severe cartilage degeneration with loss of chondrocytes and proteoglycan was seen in femoral medial condyle; synovial inflammation with severe degree of inflammatory reaction which was characterized by synovial hyperplasia, fibrosis and infiltration with a mixed mononuclear cell was seen in all synovium. These pathological changes resembled the characteristics of OA.

In recent years, much interest has centered on the role of cytokines in the pathogenesis in OA. IL-1β and TNF-α are considered to be the principal inflammatory cytokines. Sandra[38] studied in induced murine streptococcal cell wall arthritis and found that the involvement of TNF-α in this model was limited to joint swelling, whereas IL-1β played a dominant role in cartilage destruction and inflammatory cell influx. Clinical trials also revealed that the level of TNF-α and IL-1β in joint fluid of an ACL injured knee was high[39]. TNF-α neutralization substantially relieved arthritis symptoms, such as pain and number of swollen joints[40,41], and the trial with IL-1Ra suggested that IL-1 blocking results in amelioration of joint erosion[42,43]. Therefore, some researches for therapy of OA were focused on IL-1β and TNF-α. Julio[44] transferred IL-1Ra gene *in vivo* to treat rabbit knee joint OA, and Frisbie[14] delivered IL-1Ra gene *in vivo* to treat experiment equine OA. They all indicated that IL-1Ra could significantly reduce the progression of experimental OA. Seon[45] delivered IL-1Ra and soluble TNF receptor (sTNFR) into antigen-induced arthritis that antagonized the effects of IL-1β and TNF-α, and found that the co-delivery of both IL-1Ra and sTNFR resulted in a synergistic effects in disease amelioration.

NF-κB induces rapid expression of multiple genes involved in immune and inflammatory responses. It regulates

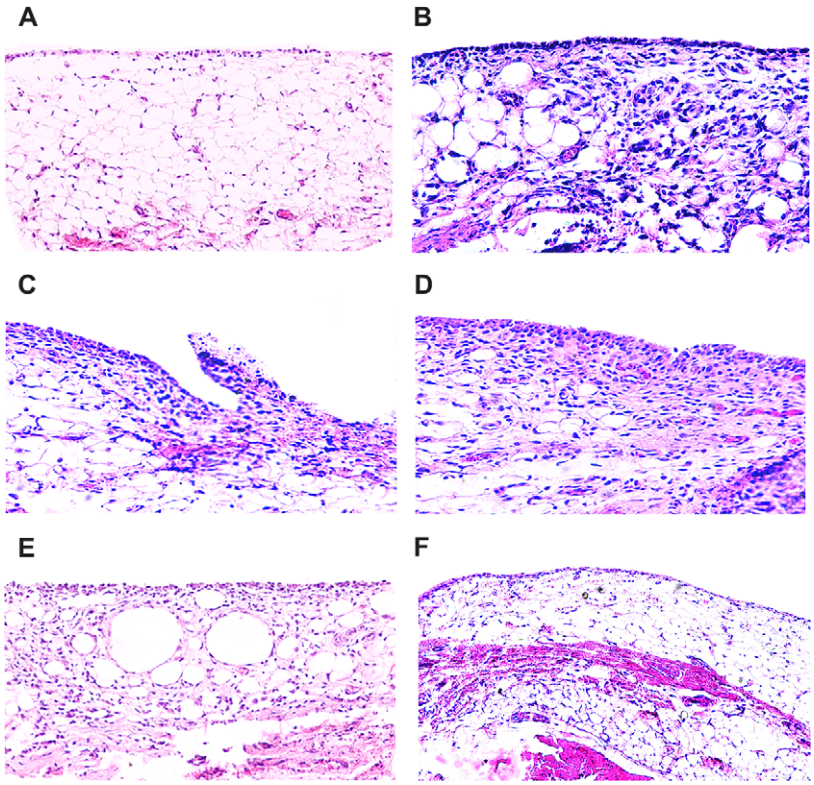

Fig. 12. The representative sections of synovium from normal (A), OA (B), OA injected with Ad-lacZ (C), OA injected with Ad-siRNA^{scrambled} (D), OA treated with aspirin (E) and OA treated with Ad-siRNA$^{NF\text{-}\kappa Bp65}$ (F) at 2 weeks after surgery with H and E staining (×200).

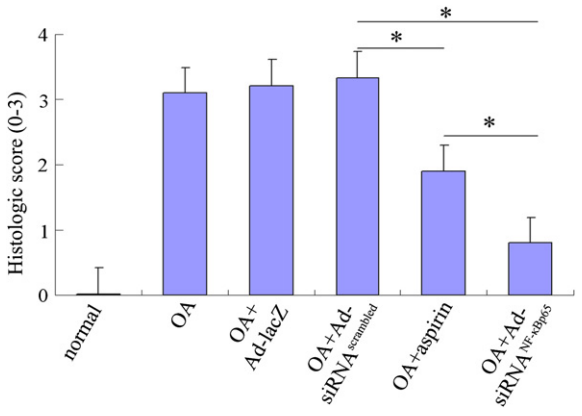

Fig. 13. Histologic grading of synovial membrane from OA joints of rats at 2 weeks after surgery. The samples were quantitatively classified according to synovium hypercellularity and the extent of synovitis. Values are the mean ± S.E.M. total score (0–3 scale) of lesions from OA rats without treatment; OA rats with intra-articular injection of Ad-lacZ; OA rats with intra-articular injection of Ad-siRNA^{scrambled}; OA rats treated with aspirin; OA rats treated with intra-articular injection of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ and rats that neither induced with arthritis nor treatment. Asterisks (*) denote values which differ at $P < 0.01$.

the expression of IL-1β and TNF-α and is also activated by these two genes, which may lead to a feed-forward amplification, with chronic activation of NF-κB in certain cells[46]. So we attempted to interrupt the signal pathway of NF-κB, cut into this feed-forward amplification and explore an approach to treat OA. Tetsuya[47] found that intra-articular transfection of NF-κB decoy oligodeoxynucleotides in arthritic joints of rats with collagen-induced arthritis using the hemagglutinating virus of Japan-liposome method led to an amelioration of arthritis. In this study, we used adenoviral vector to transfer NF-κBp65-specific siRNA into the knee joints of rat OA model and observed that Ad-siRNA could inhibit the expression of NF-κBp65 in cartilage and synovium, NF-κBp65-specific siRNA could inhibit the activation of NF-κB in cartilage, the expression of IL-1β and TNF-α in synovial fluid and suppress the progression of OA with reduction of cartilage degeneration and alleviation of synovitis. This indicated that adenoviral vector could effectively transfer siRNA into chondrocytes and synoviocytes and lead the siRNA to exert silencing effect, and NF-κBp65 played a key role in mediation of TNF-α and IL-1β.

siRNAs are short, double-stranded RNA molecules that can target mRNAs with complementary sequence for degradation *via* a cellular process termed RNA interference (RNAi)[48]. Researchers in many disciplines employ RNAi to analyze gene function in mammalian cells. The siRNAs used in early studies were typically prepared *in vitro* or by chemical synthesis and transfected into cells. Some laboratories directly used siRNA *in vivo* in studies[49–51], and they reported the use of a rapid injection method to deliver a large volume of physiological solution containing siRNAs into the tail vein of mice, and demonstrated the effectiveness of this method in reducing target gene expression in cells throughout the body. In another study, injection of liposomes containing siRNAs directed against the mRNA-encoding agouti-related peptide resulted in an increase in metabolic rate and reduced body weight without a change in food intake[52]. More recent publications feature plasmids, and expression cassettes made by PCR that include RNA polymerase promoters upstream of a hairpin siRNA template. This strategy of introducing an siRNA template into cells, and taking advantage of endogenous RNA polymerases to transcribe double-stranded RNA (dsRNA) can also be used with adenoviral vectors[25]. Upon introduction into mammalian cells, the siRNA template is transcribed, producing a 19-mer hairpin siRNA. The hairpin siRNA is recognized by Dicer, the nuclease responsible for activating dsRNAs for the RNAi pathway, and is cleaved to form functional siRNA. Adenoviral vectors are episomal, thus, siRNAs are expressed transiently, and have been extensively used to express genes in postmitotic neurons and tumor *in vivo*[53–55]. There are several reasons why viral vectors are used in basic and applied RNAi research. One major reason is that expression vectors allow continuous production of siRNAs in cells and, therefore, sustained depletion of the protein encoded by the targeted mRNA. A second reason is that the transfection efficiency of certain types of cells, particularly postmitotic cells can be greatly increased. A third advantage of viral vectors is that they are typically more effective in obtaining sustained expression and gene silencing *in vivo*[56]. Our study indicates that adenoviral vector can deliver siRNA into cells of cartilage and synovium.

Gelse[57] injected Ad-lacZ (1×10^9 pfu) into mouse knee joint and found that Ad-lacZ infected the entire joint space and positively stained cells were still found 2 weeks after the injection. Kuboki[58] injected the adenovirus suspension into the joints of guinea-pigs and did not find any hypertrophy in the superficial cells of the synovium, or marked infiltration of inflammatory cells, and no distant expression of the delivered genes observed. So we injected Ad-siRNA into rat knee joints with 1.6×10^8 pfu, and observed the changes of cytokines in synovial fluid, cartilage and synovium at 2 weeks after surgery. Although the expression of IL-1β and TNF-α and the scores of synovium and cartilage in Ad-lacZ groups were more obvious compared with OA groups, there were no significant differences between two groups. And the marked, acute synovial fluid leukocytosis was not seen at any joints.

Acetylsalicylic acid (aspirin) is a non-steroidal anti-inflammatory drug (NSAID) widely used for its anti-inflammatory, antipyretic and anti-rheumatic properties[59,60], and can prevent TNF-α- and IL-1-induced NF-κB activation in a dose-dependent manner through inhibition of phosphorylation and degradation of IκBα and IκBβ[61]. Therefore we used aspirin to treat control rats in our study, and found that aspirin could lessen the expression of TNF-α and IL-1β, and slightly alleviate synovitis and degradation of cartilage.

Since NF-κB is a known cell survival signal for most cells, we realize that chronic and long-term reduction of basal NF-κB could lead to cell death or apoptosis. However, we believe that this could still be an effective anti-inflammatory intervention under disease and stimulated conditions. Importantly, the technology of siRNA which is a gene knockdown, not a gene knockout, can only decrease the increased levels of expression of catabolic genes in the early phase of OA, but cannot completely block conduction of the signal of NF-κB. So we conclude that although the precise mechanism of siRNA remains uncertain, it is likely that adenovirus mediated NF-κBp65-specific siRNA could be developed as a powerful approach to restrain the induction of IL-1β and TNF-α, alleviate the inflammation of synovium and reduce the degradation of cartilage in early phase of OA model. Further observations on optimization of infective condition, long-term effect of Ad-siRNA$^{NF\text{-}\kappa Bp65}$ and effects on other organize are needed.

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