Adeno-associated virus vector-mediated anti-angiogenic gene therapy for collagen-induced arthritis in mice

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Abstract

Objective

The goal of this study was to determine the utility of adeno-associated virus (AAV) vectors for anti-angiogenic gene therapy in a mouse model of collagen-induced arthritis (CIA).

Methods

CIA mice were generated by immunization with bovine type-II collagen and Freund’s complete adjuvant. AAV vectors containing angiostatin and enhanced green fluorescent protein (eGFP) expression units (AAV-Ang/GFP) or the GFP and neomycin phosphotransferase (NeoR) expression units (AAV-GFP/NeoR) were injected into mouse knee joints before development of arthritis. The expression of transgenes was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and immunostaining, and the incidence and severity of arthritis was determined histologically via assessment of synovial hyperplasia, cartilage erosion and bone erosion. Vascularity in the knee joint was evaluated by immunohistochemical staining with anti-von Willebrand factor antibody.

Results

AAV vectors were capable of efficient gene transfer into chondrocytes and synovial cells, and the extent of synovial hyperplasia and other parameters of arthritis were significantly reduced in the knee joints injected with AAV-Ang/GFP compared with the joints treated with either AAV-GFP/NeoR or phosphate-buffered solution (PBS). Reduction in the number of vessels was confirmed in AAV-Ang/GFP-treated joints.

Conclusion

AAV-vector-mediated local expression of angiostatin efficiently inhibited the development of collagen-induced arthritis in the treated joint. Anti-angiogenic gene therapy using AAV vector may provide a new approach for the effective treatment of rheumatoid arthritis.

Key words

Angiostatin, adeno-associated virus vector, gene therapy, rheumatoid arthritis, collagen-induced arthritis, synovial neovascularization.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammatory synovitis and subsequent progressive destruction of articular tissues with pathologic joint lesions consisting of synovial cell hyperplasia and infiltration by various inflammatory cells. Angiogenesis play a key role in the pathology of various disease states, including diabetic retinopathy, solid tumor growth, psoriasis, scleroderma, and pannus formation in RA (1). Further, the potent angiogenic cytokine, vascular endothelial growth factor (VEGF), is up-regulated in the synovium of patients with RA (2, 3). These results suggest that angiogenesis may play an important role in hyperplasia of synovial cells and, therefore, anti-angiogenesis may represent an effective strategy for the treatment of rheumatoid arthritis.

Angiostatin is a potent endogenous anti-angiogenic factor that is derived from plasminogen and was originally purified from the serum and urine of mice with primary Lewis lung carcinoma tumors (4). Use of purified recombinant angiostatin and vectors carrying the angiostatin expression unit has been successful in the attenuation of tumor growth and secondary metastasis in various cancer models (5-7), and these effects may also prove useful in the treatment of pathologic neovascularization in various non-cancer disorders. We recently reported that HIV vector carrying an angiostatin expression unit inhibited retinal neovascularization in a mouse model of proliferative retinopathy (8) and inhibited progression of collagen-induced arthritis in mice (9).

A disadvantage of HIV based vectors is potential pathogenicity of parent virus for human species. Although the recombinant HIV vector was extensively modified to increase the safety, its clinical application is still strictly restricted. Adeno-associated virus (AAV) vectors are nonpathogenic and less immunogenic compared with other types of gene therapy vectors. The AAV genome shows stable persistence in transduced cells and achieves long-term transgene expression. Thus, AAV is an ideal gene transfer vector for use in anti-angiogenic therapy. Thus, the goal of the present study was to determine utility of AAV vectors for anti-angiogenic gene therapy of rheumatoid arthritis in a mouse model of collagen-induced arthritis (CIA).

Materials and methods

Vector production

The 1.4 kb mouse angiostatin cDNA (mAngHA), which includes a signal peptide and the first four triple loop structures (kringle regions) of plasminogen and the human influenza hemagglutinin (HA) tag was synthesized by polymerase chain reaction (PCR) using the mouse plasminogen gene (IMAGE clone no. 2225648) purchased from Incyte Genomics, Inc. (Palo Alto, CA, USA) as a template. Sense (5'-TCGTCGACATGGACCCATAAG-GAAATATCC-3') and anti-sense (5'-ACGGTGAGTTAGGGC-TAGCGTAATCCGGAACATCG-TATGGGTATGGGCAATTCACAACA-3') sequences were used as primers. AAV vector plasmids, pAAV-Ang/GFP and pAAV-GFP/NeoR, are derivatives of psub201 (10). pAAV-Ang/GFP contains mAngHA driven by the CAG promoter (11) and enhanced green fluorescence protein (eGFP) cDNA driven by the B19 promoter (12). pAAV-GFP/NeoR contains eGFP cDNA driven by the CAG promoter and neomycin phosphotransferase (NeoR) expression unit from pMC1neo (13).

Recombinant AAV vectors subtype 2, AAV-Ang/GFP and AAV-GFP/NeoR were generated by the adenovirus-dependent classical transfection-infection method and concentrated by the combination of sulfonated-cellulose column chromatography (Seikagaku Kougyo, Tokyo) and ultraltration using Centriprep (Millipore Corp. Billerica, MA, USA). All vector stocks were assayed for adenovirus contamination via a plaque assay on permissive HeLa cells and were completely free of adenovirus. The titer of AAV vectors was determined by slot-blot hybridization assay. The titer of final preparation of each AAV vector was 2.0-3.7 x 10^{12} AAV particles per ml.
Western blot
HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). HeLa cells were plated in six-well plates (2 x 10^5 cells/well) and transduced with AAV vector (3.7 x 10^11 particles) in 1 ml of DMEM with 0.1% (v/v) FBS. Three days after transduction, the conditioned medium was concentrated to 1/40th of its original volume by ultrafiltration with a Microcon (Millipore Corporation, Bedford, MA, USA). The transduced HeLa cells were rinsed with phosphate-buffered saline (PBS), then lysed in mammalian lysis buffer (C-2978, CellLytic™-M, SIGMA) at 4°C for 30 min, and insoluble materials were removed from the lysate by centrifugation at 15,000g for 10 minutes. Anti-angiostatin antibody bound Protein G Sepharose was prepared by incubating 1 µg of the anti-angiostatin polyclonal antibody (Y’410, Yanaihara Institute inc, Shizuoka, Japan) with 20 µl of 50% (v/v) Protein G-Sepharose Fast Flow Slurry (Pharmacia Biotech AB, Uppsala, Sweden) in Tris-based saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature on an end-over-end rotator, followed by extensive washing with TBST. The cell lysate and the concentrated conditioned medium was immunoprecipitated with the anti-angiostatin antibody-bound Protein G Sepharose at 4°C for 1 h. Immunoprecipitates were collected by brief centrifugation and were washed four times with 200 µl of lysis buffer. The immunoprecipitated proteins were boiled for 2 minutes in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer including mercaptoethanol. After brief centrifugation, the supernatants were resolved by SDS-PAGE, then electrophoblotted onto an Immobilon-P membrane (Millipore Corp. Billerica, MA, USA) at 30 V for 18 h or at 60 V for 2 h. The blots were blocked with 5% low-fat milk in TBST for 1 h at room temperature, washed three times with TBST containing Tween-20 (1:2,000), and incubated with the appropriate concentration (0.5 µg/ml) of anti-HA antibodies (mouse monoclonal antibody clone 12CA5, Roche) at 4°C for 1 h. After washing three times with TBST, the blots were probed with a 1:10,000 dilution of goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA) for 60 min at room temperature. After the third wash, immunoreactive proteins were detected using an enhanced chemiluminescent detection system according to the manufacturer’s directions (ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK). The relative intensities of the protein bands were quantified by scanning densitometry.

Preparation of CIA mice and experimental protocols
All animal experiments were conducted in accordance with the regulations of the Ethical Committee of Nippon Medical School. CIA mice were prepared as described previously (9). Female DBA/1J mice (5 weeks of age) were immunized intradermally at the base of the tail with 100 µl of the emulsion containing 100 µg of bovine type-II collagen and Freund’s complete adjuvant. The same injection was repeated as the booster. Severe arthritis developed in all joints between 3 and 4 weeks after the booster under these experimental conditions (14). In the treatment experiments, the AAV vector was injected at the same time as the booster, prior to establishment of arthritis. Five microliters of AAV-Ang/GFP or AAV-GFP/NeoR (1.0-1.9 x 10^10 particles) was injected into the right knee joint space using a Hamilton syringe with a 30 gauge needle. PBS (5 µl) was injected into the left joints as a control. Mice were sacrificed at 4 and 8 weeks after vector injection.

RNA analysis
Total RNA was isolated from the knee joint for reverse transcriptase (RT)-PCR as described previously (9). The eGFP forward primer was 5’-CATCCTGGTGCGACTTGACG-3’ and the eGFP reverse primer was 5’-GACTGGGTGCTAGGTAGTG-3’. These primers amplify 576 bp of eGFP mRNA derived from AAV-ang/GFP vector. Further, PCR primers for mouse β-actin mRNA (control) and the conditions for PCR were identical to those reports (8, 9). The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed.

Histological assessment of arthritis
Whole knee joints were removed and fixed for 4 days in 10% formaldehyde. After decalcification in 5% formic acid, the specimens were embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin (HE staining). Histological assessment was classified on a scale of 0 to 4+ (15). Histological changes in the intra-articular tissues were scored using following parameters: synovial hyperplasia (0 to 4+), cartilage erosion (0 to 4+) and bone erosion (0 to 4+). These joint scores were given equal weight in determining the final joint score, and the final joint score was divided by the number of parameters (e.g., 3) to arrive at a maximal joint severity score of (4+4+4)/3 = 4.

Immunohistochemical detection
Expression of eGFP in the synovial tissues was examined as described previously (9). Detection of HA-tagged angiostatin was performed using the same protocol and anti-HA antibody (clone 16B12, BAbCO). Synovial vessels in the knee joint were identified by immunohistochemistry with a rabbit polyclonal antibody against von Willebrand Factor (CODE NO.: N1505, DAKO CO). Thin-walled, capillary-like vessels in the high power field (x 400) sections were counted.

Statistical analysis
Statistical analysis was performed using the Mann-Whitney U-test, and p <
0.05 was considered to denote statistical significance.

**Results**

*Intraarticular injection of AAV-angiostatin/GFP*

An AAV vector containing the expression units for mouse angiostatin-HA chimeric protein and eGFP (AAV-Ang/GFP) induced expression and secretion of angiostatin, as detected by Western analysis of the cell lysate and conditioned medium of transduced HeLa cells (Fig. 1A). Biological activity of angiostatin was confirmed by a tube formation assay using human umbilical vein endothelial cells (HUVECs) co-cultured with human fibroblasts (8) (data not shown).

Distribution of the AAV vector was examined by detection of vector-specific eGFP expression 4 weeks after AAV-Ang/GFP injection. RT-PCR analysis revealed that expression of eGFP RNA was localized to the right knee joint but not to other joints (Fig. 1B). Transgene expression in the knee joint was further studied by immunohistochemical analysis. Expression of eGFP was primarily detected in chondrocytes and hyperplastic synovial cells (Fig. 2). A similar staining pattern was also observed with the anti-HA antibody used to detect angiostatin-HA chimeric protein (Fig. 2). These results indicate that intraarticular injection of AAV vector induced local expression of the transgene in the joint.

*Effects of AAV-mediated expression of angiostatin on arthritis in CIA mice*

To investigate the therapeutic effects of the AAV vector, CIA mice were injected with 5 µl of the AAV-Ang/GFP vector or the control AAV-GFP/Neo vector in the right knee joint and were injected with 5 µl of PBS in the left knee joint. Histological evaluation for arthritis was performed 4 and 8 weeks later. Severe arthritis, characterized by marked synovial cell hyperplasia, infiltration with mononuclear cells, pannus formation and cartilage destruction, developed in knee joints injected with PBS or the control vector 8 weeks after the booster injection, whereas these inflammatory changes were significantly

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**Fig. 1.** (A) AAV-mediated expression of angiostatin. Expression and secretion of angiostatin-hemagglutinin (HA) was confirmed by western blotting. Cell lysates from HeLa cells without treatment (lane 1), transfected with pAAV-Ang/GFP (lane 2), transduced with AAV-Ang/GFP (lane 3) and conditioned medium from HeLa cells transduced AAV-Ang/GFP (lane 4, 5) were immunoprecipitated with the anti-angiostatin antibody followed by western blotting with the anti-HA antibody. In lane 5, the medium was concentrated by a factor of 40 using Microcon before immunoprecipitation.

(B) Distribution of AAV vector after intraarticular injection. Total RNA extracted from the right knee joint (lane 1), left knee joint (lane 2), heart (lane 3), kidney (lane 4), liver (lane 5) and lung (lane 6) of CIA mice 4 weeks after injection of AAV-Ang/GFP into the right knee joint was analyzed by RT-PCR.

**Fig. 2.** Detection of AAV mediated transgene expression in the right knee joints. Synovium and cartilage from the right knee joints of collagen-induced arthritis mice 4 weeks after injection of AAV-Ang/GFP were immunostained with anti-eGFP(middle panels) and anti-HA(bottom panels) antibodies, followed by peroxidase-conjugated anti-mouse IgG. Top panels show negative control immunostained only with the second antibody.
suppressed in the joint treated with AA V-Ang/GFP vector (Fig. 3). A significant decrease in knee joint score was observed at 4 and 8 weeks after vector injection in the knee joints that received the AA V-Ang/GFP vector (Fig. 5A). These results indicate that local treatment with AA V vector encoding the angiostatin gene resulted in reduction of knee joint disease in CIA mice.

To study the mechanism of AA V-mediated inhibition of CIA development, vascularity of the knee joints was assessed by immunostaining with anti-von Willebrand Factor antibody (Fig. 4). The number of vessels was significantly decreased in the treated joint, suggesting that the AA V vector produced angiostatin in levels that were biologically active (Fig. 5B). In several experimental systems, suppression of arthritis was observed in non-injected joints as well as injected joints after local administration of viral vector, although the mechanism of this distant effect is not well understood (18-20). Macroscopic assessment of the hind paw for signs of arthritis at 4 and 8 weeks after vector injection was performed, but no changes in ipsilateral joints were detected (Fig. 5C).

**Discussion**

This study demonstrated that local injection of the AA V vector carrying the angiostatin expression unit efficiently inhibited development of collagen-induced arthritis. AA V vectors were able to transduce and induce transgene expression in chondrocytes and synovial cells without provoking synovial inflammation. This strategy was successful in accumulation of anti-arthritic proteins in the articular tissues, and reduce the exposure of therapeutic protein to extra-articular tissues and organs, thereby reducing potential side effects associated with systemic therapy (21).

We recently reported that HIV vector mediated expression of angiostatin inhibited the progression of disease in CIA mice (9). The present study further supports the utility of angiogenic gene therapy approach for treatment of rheumatoid arthritis. The most important point of the present study is that we used clinically applicable AA V vectors. HIV vectors have been shown to be useful for long-term expression of transgene in various experimental animals. However, the clinical use of HIV vector is hampered by its safety concern. The advantages of AA V vectors include nonpathogenicity and long-term stable expression of transgenes without chromosomal integration. The safety of AA V vectors have been confirmed by clinical trials (Hemophilia). Therefore, gene therapy of RA using AA V vectors is highly realistic.

Angiostatin is an endogenous inhibitor of angiogenesis that inhibits endothelial cell migration and induces endothelial-cell-specific apoptosis (22) by mechanisms that remain unclear. Recently, studies have reported identification of various angiostatin binding proteins, including alpha/beta subunits of ATP synthase (23), alphavbeta3 integrins (24), and angiomotin (25). Kim et al. suggested that retroviral-mediated ex
vivo anti-angiogenic cell therapy with angiostatin may be of benefit for RA (26), while we demonstrated that HIV mediated intraarticular expression of angiostatin inhibited the progression of disease in CIA mice. These reports, taken together with data from the present study, suggest that local expression of angiostatin is a promising approach for the treatment of RA.

We used adeno-associated virus vector for anti-angiogenic gene therapy of arthritis in this study. Adenoviral gene transfer was shown to be well suited for gene therapy in mouse models of arthritis because of the high efficiency of in vivo gene transfer into synovial cells and inflammatory cells. However, adenoviral vectors also stimulate a host immune response that may shorten the longevity of modulated target cells. Unlike adenoviral vectors, AAV vectors do not encode for viral proteins that may induce host immune responses (27,28). Thus, the AAV vector appears most clinically useful for the local treatment of chronic inflammation. The AAV vector has been previously used to treat a tumor necrosis factor-α model of mouse arthritis (15). According to this study, AAV-mediated expression of soluble TNF-α receptors reached maximal levels between 3 and 4 weeks and was still detectable at 60 days after vector injection in the knee joint. Because of the lack of a sensitive assay system, the detailed time course of angiostatin expression could not be studied in our system. However, expression of angiostatin from the AAV vector in the joint tissue of CIA model mice was clearly detected at 4 and 8 weeks post injection by immunohistochemical assay. These results indicate that the AAV vector can achieve long term transgene expression in the joint tissues from different arthritic model mice.

Several reports demonstrated that intraarticular or periarticular administration of immunoregulatory genes suppressed arthritis in the injected site as well as in distant non-injected joints (18-20). While the basis of this distant effect is not clear, leakage of the vector and/or the transgene product from the injected site may contribute to this phenomenon. Alternatively, Evans and colleagues suggested that transduced cells migrate from injected to uninjected joints (29). Ipsilateral effects were also observed when using HIV vector encoding the angiostatin gene. In contrast, distant effects were not apparent in the present study using AAV vector. The differences in cell specificity and transduction efficiency between two vector systems may explain this apparent discrepancy. Strict localization of AAV-mediated gene expression and its biological effect to treated joints will likely result in a low incidence of systemic side effects.

**Fig. 5.** Quantitative evaluation of the joints after anti-angiogenic gene therapy. (A) Histological changes of the knee joints. Synovial hyperplasia, cartilage erosion, and bone erosion were scored by the method described in Materials and methods. **Solid bars:** the left joint injected with PBS. **Open bars:** the right joint injected with AAV-Ang/GFP. **Gray bars:** the left joint injected with PBS. **Hatched bars:** the right joint injected with AAV-GFP/NeoR. (B) The number of vessels in the knee joints. Thin walled, capillary-like vessels were counted after immunostaining with an anti-von Willbrand factor antibody. (C) Macroscopic changes in the hind paws. Swelling, redness, and presence of edema of the hind paws were evaluated after treatment of knee joints by the method described in Materials and Methods. Values are the mean SEM (n = 6–16). *p < 0.05. n.s, not significant.
In summary, injection of AAV-angiostatin into the joint significantly inhibited disease development in a mouse model of collagen-induced arthritis without affecting untreated joints. Thus, local delivery of AAV represents a promising strategy for the treatment of RA.

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