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ADENO-ASSOCIATED VECTOR MEDIATED GENE TRANSFER OF TRANSFORMING GROWTH FACTOR – β1 TO NORMAL AND OSTEOARTHRITIC HUMAN CHONDROCYTES STIMULATES CARTILAGE ANABOLISM

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Abstract

The objective of the present study was to investigate whether cartilage anabolism in human primary osteoarthritic chondrocytes could be improved by adeno-associated virus (AAV) vector-mediated gene transduction of transforming growth factor TGF-β1 (TGF-β1).

A bi-cistronic AAV-TGF-β1-IRES-eGFP (AAV-TGF-β1) vector was generated and used for transduction of a normal human articular chondrocyte cell line (tsT/AC62) and primary human osteoarthritic articular chondrocytes harvested from 8 patients receiving total knee joint arthroplasty. Transduction efficiency was detected by fluorescent microscopy for gene expression of enhanced green fluorescent protein (eGFP). TGF-β1 synthesis was determined by ELISA. To assess the influence of TGF-β1 gene therapy on chondrocyte cartilage metabolism, mRNA expressions of type II collagen, aggrecan, and matrix metalloproteinase 3 (MMP-3) were determined by quantitative real-time PCR.

AAV-TGF-β1 transduction resulted in increased synthesis of TGF-β1 in both osteoarthritic chondrocytes and the normal articular chondrocyte cell line. The expression levels of the transduced genes were correlated to "multiplicity of infection" (MOI) and post-infectious time. In both osteoarthritic chondrocytes and the normal articular chondrocyte cell line, AAV-TGF-β1 treatment increased mRNA expression of both type II collagen and aggrecan, but decreased MMP-3 mRNA expression. Osteoarthritic chondrocytes and the normal articular chondrocyte cell line could be transduced with equal efficiencies.

In conclusion, it was demonstrated that AAV-TGF-β1 gene transfer stimulates cartilage anabolism and decreases expression of enzymes responsible for cartilage degradation in human osteoarthritic chondrocytes. The results indicate that the AAV vector is an efficient mediator of growth factors to human articular chondrocytes, and that it might be useful in future chondrocyte gene therapy.

Keywords: Adeno-Associated Virus, Transforming Growth Factor-β1, gene therapy, cartilage, osteoarthritis

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Introduction

Articular cartilage injury (Alleyne *et al.*, 2001; Browne *et al.*, 2000; Kish *et al.*, 1999; Martinek *et al.*,2001), and osteoarthritis (Frenkel *et al.*,1999; Malemud *et al.*, 1999; Vangsness, 1999) remain serious clinical problems and, collectively, are among the most prevalent pathologies that affect human joints. Even though, the presence of mesenchymal stem-cells in articular cartilage has recently been reported (Alsalameh *et al.*, 2004; Dowthwaite *et al.*, 2004), the cartilage repair response to injury and osteoarthritis is inadequate (Buckwalter *et al.*, 1998; Ulrich-Vinther *et al.*, 2003). Hence, stimulation of deteriorated cartilage with growth factors may be attractive. Various studies have demonstrated that transforming growth factor TGF-β1 (TGF-β1) is one of the most potent of the various growth factors for cartilage repair (Grimaud *et al.*, 2002). TGF-β1 directly stimulates proteoglycan and collagen synthesis (Redini *et al.*, 1988) and antagonizes the effects of IL-1 on matrix metalloproteinases (MMP) in normal and osteoarthritic chondrocytes (Andrews *et al.*, 1989; Lum *et al.*, 1996; van Beuningen *et al.*, 2000; van der Kraan *et al.*, 2000).

As cartilage damage and osteoarthritis affect a limited number of large, weight-bearing joints and have no major extra-articular manifestations, they might be well suited for local, intra-articular gene therapy by which the chondrocytes can be long-term stimulated by chondro-anabolic growth factors such as TGF-β1. Previous studies have reported gene delivery into human chondrocytes with the use of naked DNA and viral vectors (Arai *et al.*, 1997; Baragi *et al.*, 1997; Doherty *et al.*, 1998; Kang *et al.*, 1997). However, problems with these gene transfer methods are related to the ability to obtain high-efficiency transduction, to maintain long-term expression of the therapeutic gene, and appropriate safety profiles of the vector systems. Recently, vectors based on the adeno-associated virus (AAV) have convincingly mediated transduction of reporter genes to human articular chondrocytes (Arai *et al.*, 2000; Madry *et al.*, 2003; Ulrich-Vinther *et al.*, 2002). These studies have identified that the AAV vector is capable of efficient gene transfer and sustained gene expression in both normal primary articular chondrocyte cultures, and articular cartilage explants, and direct *in vivo* gene therapy (Ulrich-Vinther *et al.*, 2004). Furthermore, gene therapy with AAV has several important advantages, including an attractive vector safety profile, and the ability of long-term expression in tissues with low mitotic rate (Schwarz, 2000).

Therefore, we now have tested the potency of AAV-TGF-β1 gene transfer to increase cartilage anabolism in human articular chondrocytes derived from both normal articular cartilage and osteoarthritic joints from 8 human patients.

Materials and Methods

Preparation of the AAV-TGF-β1-IRES-eGFP vector

Generation of the bi-cistronic rAAV type 2 transfer vector, rAAV-TGF-β1-IRES-eGFP, was carried out *via* multiple subcloning steps as described previously (Ito *et al*., 2004; Ulrich-Vinther *et al*., 2002). In brief, the rAAV-TGF-β1-IRES-eGFP vector was generated by replacing the OPG cDNA in the pAAV-OPG-IRES-eGFP (Ulrich-Vinther *et al*., 2002) with a porcine TGF-β1 cDNA fragment that was subcloned into the *Not*I and *Eco*RI sites. The bi-cistronic insert was under transcriptional control of a CMV promoter. Extensive restriction digests and double-stranded cDNA sequencing of the TGF-β1 cDNA were performed to confirm the authenticity of the vector. Subsequently, 0.5 mg of pAAV-TGF-β1-IRES-eGFP was sent to the Gene Core Facility, University of North Carolina at Chapel Hill, NC, which prepared the purified rAAV-TGF-β1-IRES-eGFP, and also provided the rAAV-eGFP control vector using a helper virus free method (Xiao *et al*., 1998). The concentration of infectious rAAV-TGF-β1-IRES-eGFP units was approximately $4x10^{12}$ infectious particles per mL determined by titration on human embryonic kidney 293 cells (Goater *et al*., 2002; Ulrich-Vinther *et al*., 2002).

Human articular chondrocyte cultures

Due to the limited access to normal human articular cartilage for primary cellular studies, the well-characterized normal adult human articular chondrocyte cell line, tsT/AC62, immortalized with a temperature-sensitive mutant of SV40 large T antigen (Robbins *et al*., 2000), was used. The tsT/AC62 cells were cultured routinely for proliferation in monolayer cultures at 32°C in a humidified sterile atmosphere of 95% air and 5% CO_2. The cells were passaged every 7-10 days at a split ratio of 1:2. For transduction experiments, the tsT/AC62 cells were plated in 6-well tissue culture plates at a density of 10^6 cells per well (~80% confluence) in Dulbecco's modified Eagle's medium (DMEM) / Ham's F-12 (1:1) (Gibco BRL, Gaithersburg, MD, USA) at an increased temperature of 37°C in a humidified sterile atmosphere of 95% air and 5% CO_2. The tsT/AC62 cell cultures were transduced 24 hours after plating.

Additionally, osteoarthritic articular cartilage was harvested from the knee joints of 8 patients undergoing total knee arthroplasty surgery due to severe primary osteoarthritis. The population was constituted of 4 females and 4 males with a mean group age of 66.4 years ± 3.1 years (mean ± SEM). Beside paracetamol, the patients did not receive any medicine. The Danish Ethical Committee approved the use of human cartilage, and informed consent was obtained from all patients included. The cartilage specimens from the 8 patients were kept separately for individual gene transductions, cultures and paired analyses. Immediately after harvesting, the articular cartilage specimens from the 8 patients were carefully dissected from the subchondral bone, and finely chopped. The fragments were washed twice with sterile phosphate buffered saline. The chondrocytes were liberated from the extra-cellular matrix by a two-step enzymatic digestion including a brief hyaluronidase digestion with 1% testicular hyaluronidase (Sigma, St. Louis, MO, USA) in DMEM/Ham's F-12 for 1 hour, and a prolonged collagenase digestion with 1% clostridial collagenase A (Sigma, St. Louis, MO, USA) in DMEM/Ham's F-12 for 24 hours. These digestions were performed under vigorous shaking at 37°C. The isolated chondrocytes were filtered (Swinnex filter with 40 mm pores (Millipore, Bedford, MA, USA)), and resuspended in DMEM/Ham's F-12 (1:1). The chondrocytes were seeded onto 6-well plastic plates at a density of 10^6 cells per well and cultured in Dulbecco's modified Eagle's medium (DMEM) / Ham's F-12 (1:1) at 37°C in a humidified sterile atmosphere of 95% air and 5% CO_2. The cultures were transduced 24 hours after plating.

Maintenance of the chondrocyte differentiation during the experiments was confirmed by reverse transcription-PCR for markers of articular chondrocyte maturity (aggrecan and types I, II, and X collagen).

Detection of transduction efficiency

The human articular chondrocyte cultures were transduced with AAV-TGF-β1-IRES-eGFP at MOIs of either 250, or 1000. Control cultures consisted of uninfected cultures and cultures infected with the AAV-eGFP vector at a MOI of 1000.

At days 0, 2, 4, 6, and 8 after transduction, the transduction efficiency of the AAV-TGF-β1-IRES-eGFP vector was determined as the relative population of green fluorescent chondrocytes in the cultures by fluorescent microscopy. Determinations of the relative population of green fluorescent chondrocytes were based counts of 250 chondrocytes. Uninfected articular chondrocytes were analyzed simultaneously and confirmed non-fluorescent.

Detection of TGF-β1 synthesis

At days 0, 2, 4, 6, and 8 after transduction, concentrations of active TGF-β1 in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA), using the porcine ELISA kit "TGF-β1 Duo Set" with TGF-TGF-β1 protein standards (Quantikine, R&D Systems, Minneapolis, MN, USA). The corresponding total concentrations of TGF-β1 (latent + active) were determined with ELISA by activation of the latent TGF-β1 to the immunoreactive form by acid activation according to manufactures instructions (Quantikine, R&D Systems, Minneapolis, MN, USA). Uninfected and AAV-eGFP transduced primary human articular chondrocytes and tsT/AC62 chondrocytes served as negative controls.

Detection of TGF-β1, type II collagen, aggrecan and MMP-3 expression

At 8 days after transduction, total RNA was extracted using

Table 1. Primer sequences used for RT-PCR amplification of TGF-β1, type II collagen, aggrecan, and MMP-3

Gene		Primer	Size (bp)
TGF-beta1	*s*	5' GAGCAGCACGTGGAGCT 3'	108
	as	5' CAACTCCGGTGACATCAAA 3'	
Type II collagen	*s*	5' GGACTTTTCTCCCCTCTCT 3'	113
	as	5' GACCCGAAGGTCTTACAGGA 3'	
Aggrecan	*s*	5' TCGAGGACAGCGAGGCC 3'	94
	as	5' TCGAGGGTGTAGCGTGTAGAGA 3'	
MMP-3	*s*	5' TGGTACCCACGGAACCTGTC 3'	59
	as	5' GATCACAGTTGGCTGGCGTC 3'	

s: sense; *as:* anti-sense

the RNeasy mini kit with the RNAse free DNase on column option (QIAGEN Inc., Venlo, Netherlands) and single-stranded cDNA was made using a reverse transcription kit (Bio-Rad iScript cDNA synthesis Kit, Bio-Rad Laboratories, CA, USA) according to manufactures instructions. The total RNA concentration was determined using Quant-It™ Ribogreen Kit (Molecular Probes Inc, OR, USA).

Real-time Polymerase Chain Reaction (PCR) was performed on the Stratagene MX4000 real-time amplification system using Stratagene Brilliant SYBR Green (Stratagene, CA, USA) according to the manufacturer's instructions. A cDNA equivalent to 6 ng of total RNA was used in each reaction. Primers for TGF-β1 and MMP3 were designed using Primer3 Software. Primer sequences for Type II collagen and aggrecan were kindly supplied by Jeff Goater, University of Rochester Medical Center, NY, USA (Table 1). Primers were manufactured by DNA-Technology, Aarhus, Denmark. For PCR, the primer concentration was 600 nM in all reaction mix. All primers were optimised with regards to primer concentration, $MgCl_2$ concentration and efficiency to achieve efficiency of 100% (Table 1).

The relative gene expression is a relative quantification in real-time PCR of a target gene transcript (AAV-TGF-β1 transduced) in comparison to a reference gene transcript (Untransduced / AAV-GFP transduced). Hence, an effect of AAV-TGF-β1 transduction on a target gene expression such as type II collagen, will be emphasized. The mean threshold cycle (Ct) values from duplicate measurements were used to calculate the relative gene expression according to Pfaffl (Pfaffl, 2001) as:

$$\text{Relative expression} = E^{\Delta Ct \text{ target (control – sample)}}$$

…with E = 2 and with normalization to the total RNA concentration according to Bustin (Bustin, 2000) as internal control.

Uninfected and AAV-eGFP transduced primary human articular chondrocytes and tsT/AC62 chondrocytes served as negative controls.

Statistical analysis

The study was designed as a paired interventional experiment. All data acquisition and analyses were performed blindly. Cartilage samples from eight patients were harvested and analyzed separately. For each parameter, measurements were performed in doublets. The data confirmed proximities to normal distribution and homogeneity of variances and, hence, parametric analyses were applied (Students paired *t*-test). Statistical significance was determined as $p < 0.05$ (two-tailed).

Results

Transduction efficiency

The AAV-TGF-β1 vector convincingly mediated reporter gene delivery to both tsT/AC62 chondrocytes and osteoarthritic chondrocytes (Fig.1,A-B). From day 2 to day 8 after transduction, the percentage of eGFP expressing cells increased in all transduced groups (tsT/AC62 chondrocytes: MOI 250; 487% ($p<0.001$), MOI 1000; 471% ($p<0.001$). Osteoarthritic chondrocytes: MOI 250; 583% ($p<0.001$), MOI 1000; 649% ($p<0.001$)) (Fig.1,B). The transduction efficiencies were positively correlated to the MOIs (Fig.1,B). Cultures of tsT/AC62 chondrocytes and osteoarthritic chondrocytes were found to be equally transduced by the AAV vector (Fig. 1,B).

Synthesis of TGF-β1

The results of TGF-β1 ELISA-measurements are depicted in Fig. 2. Throughout the observation period, administration of AAV-TGF-β1 resulted in increasing concentrations of both total and active TGF-β1 in all groups (Fig. 2). Additionally, the level of TGF-β1 synthesis depended on the MOI of AAV-TGF-β1 in both tsT/AC62 chondrocytes and osteoarthritic chondrocytes. Eight days after transduction, infection of tsT/AC62 chondrocytes at a MOI of 1000 resulted in 123 ± 11 ng/mL of total TGF-β1 and 12 ± 1 ng/mL of active TGF-β1, whereas transduction at a MOI of 250 resulted in 46 ± 5 ng/mL of total TGF-β1 and 5 ± 1 ng/mL of active

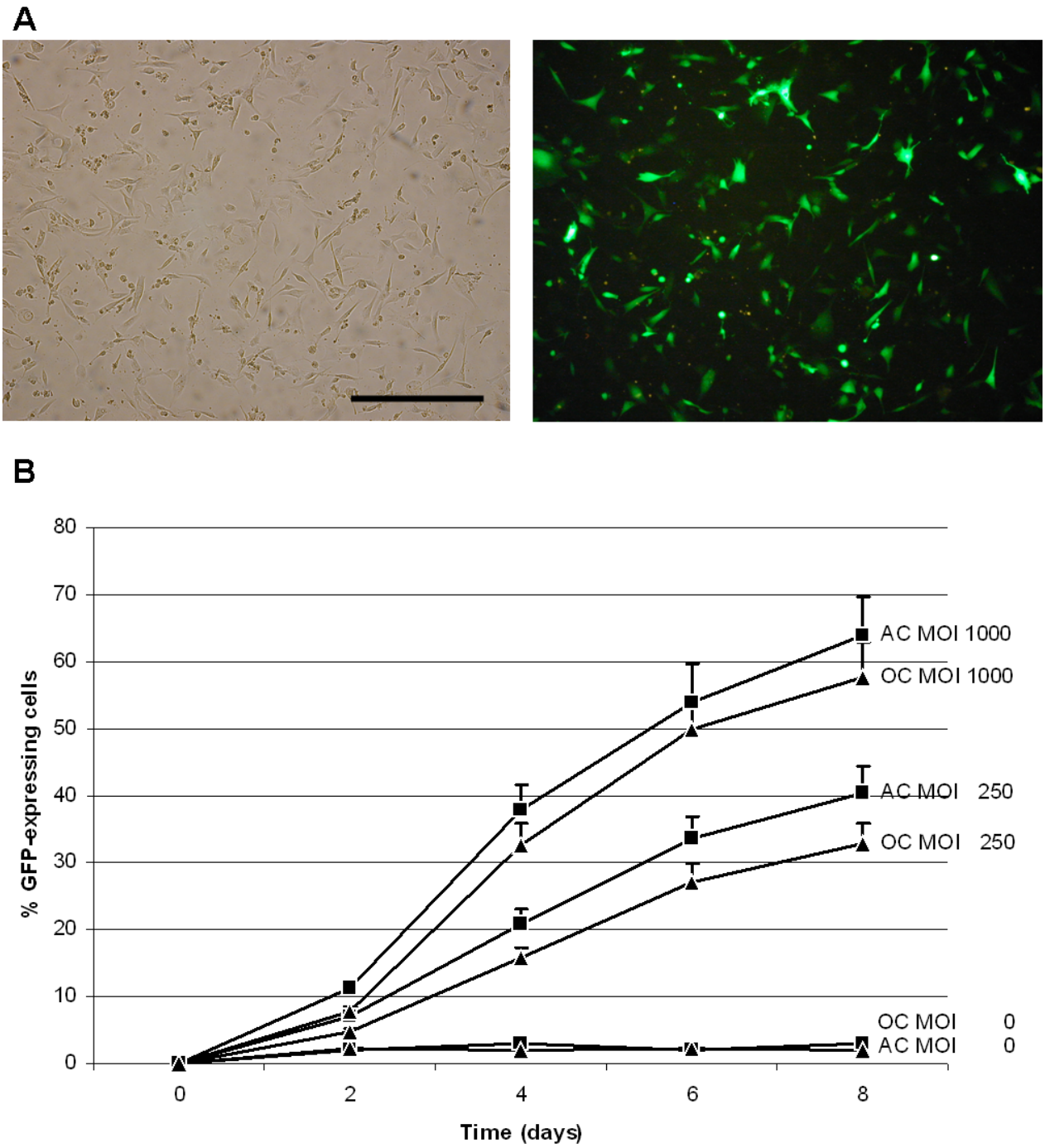

Figure 1 (A) Bright-field light (left) and fluorescence (right) microscopy of corresponding human osteoarthritic chondrocytes 8 days after AAV-TGF-β1-IRES-eGFP transduction at a MOI of 1000. Dimensions are given by scale bar (100 mm). (B) AAV-TGF-β1-IRES-eGFP transduction of both osteoarthritic human chondrocytes and a human normal articular chondrocyte cell line resulted in an increasing number of eGFP expressing chondrocytes during the observation period (mean with SEM).
AC: normal human articular chondrocyte cell line. OC: human osteoarthritic chondrocytes.
MOI: multiplicity of infection.

TGF-β1 (p (MOI 250 versus MOI 1000) < 0.05 for both total and active TGF-β1). No difference in TGF-β1 synthesis between tsT/AC62 chondrocytes and osteoarthritic chondrocytes was observed in any groups. No difference in TGF-β1 concentrations in media from uninfected chondrocytes and AAV-eGFP transduced chondrocytes was observed (data not shown).

Expression of TGF-β1, type II collagen, aggrecan, and MMP-3

The results of quantitative RT-PCR performed on extracted mRNA from articular chondrocytes at 8 days after transduction are given as cycle threshold (Ct) in Fig. 3 and as relative gene expression in Table 2.

AAV-TGF-β1 transduction of both tsT/AC62 chondrocytes and osteoarthritic chondrocytes resulted in

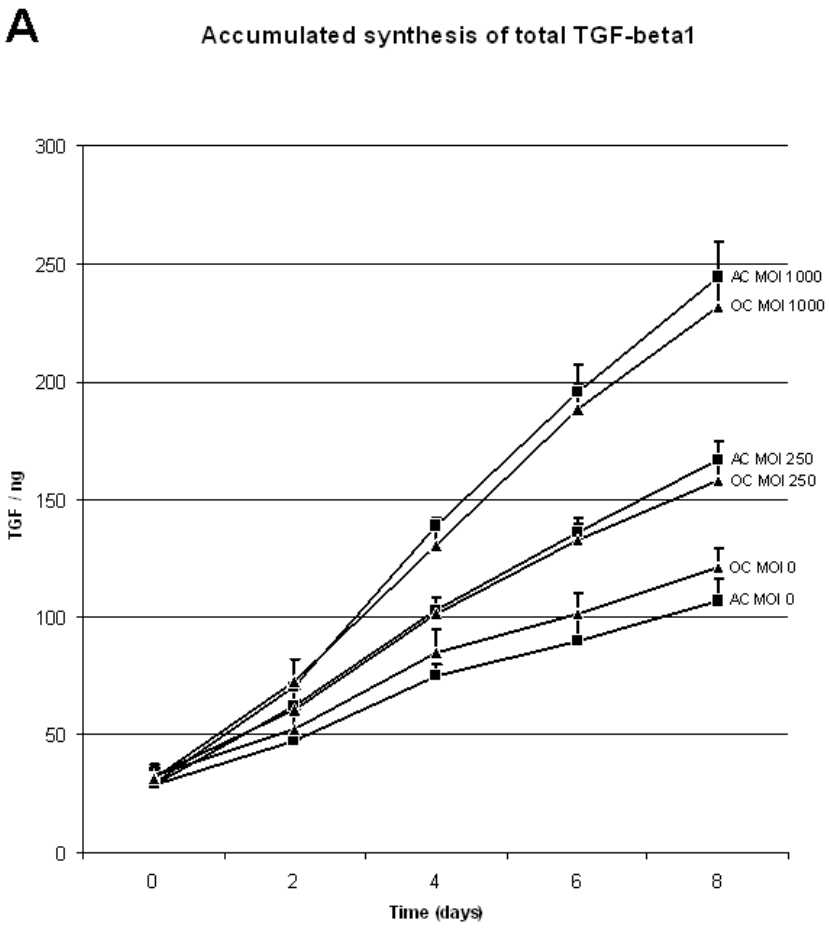

Figure 2 Synthesis of TGF-β1 in primary osteoarthritic human chondrocytes and a normal human articular chondrocyte cell line following AAV-TGF-β1 transduction. The synthesis of both latent (A) and active (B) TGF-β1 in culture media from articular chondrocytes was determined by ELISA at days 0, 2, 4, 6, and 8 after transduction (mean with SEM). All cultures infected with AAV-TGF-β1 produced significant amounts of TGF-β1 compared with the controls (not transduced and AAV-eGFP transduced cells) ($p<0.05$).

AC: normal human articular chondrocyte cell line. OC: human osteoarthritic chondrocytes.
MOI: multiplicity of infection.

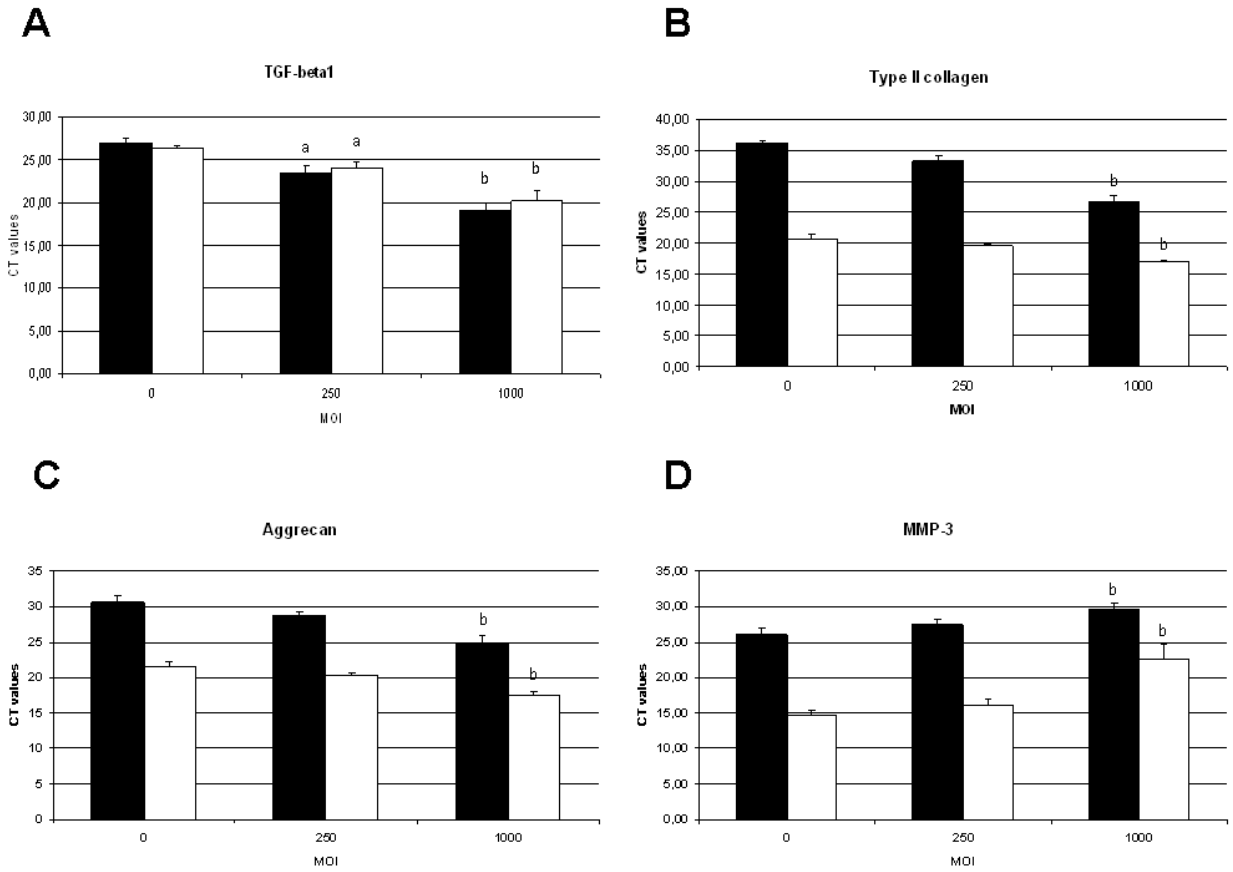

Figure 3 Cellular expression of TGF-β1, type II collagen, aggrecan, and MMP-3 in osteoarthritic human chondrocytes and a normal human articular chondrocyte cell line following AAV-TGF-β1 transduction. At day eight after transduction, total RNA was extracted and analysed by RT-PCR as described in the Material and Methods section (mean with SEM). AAV vector mediated TGF-β1 gene delivery resulted in increased expressions of TGF-β1, type II collagen, and aggrecan, but decreased expression of MMP-3 ($p<0.05$).

■ : values from a normal human articular chondrocyte cell line.

□ : values from human osteoarthritic chondrocytes.

a: Significantly different from control chondrocytes (not transduced and AAV-eGFP transduced) at MOI of 250 AAV-TGF-β1 ($p<0.05$).

b: Significantly different from control chondrocytes (not transduced and AAV-eGFP transduced) at MOI of 1000 AAV-TGF-β1 ($p<0.05$).

increased TGF-β1 mRNA expression levels matching the results of TGF-β1 synthesis determined by ELISA. Hence, TGF-β1 expression was increased by application of higher MOI of AAV-TGF-β1. No difference was found in TGF-β1 expression between tsT/AC62 chondrocytes and osteoarthritic chondrocytes (Fig. 3,A).

In tsT/AC62 chondrocytes, treatment with AAV-TGF-β1 resulted in 26% ($p<0.022$) higher expression level (Ct) of type II collagen at a MOI of 1000 compared with uninfected chondrocytes. At a MOI of 250, only an insignificant 8% ($p=0.063$) increase in expression level (Ct) of type II collagen could be observed. The relative gene expressions of type II collagen in tsT/AC62 chondrocytes were 9.3 at a MOI of 250, and 113.6 at a MOI of 1000 (Table 2). Similarly, in osteoarthritic chondrocytes, AAV-TGF-β1 transduction increased the expression level (Ct) of type II collagen mRNA by 18% ($p=0.012$) at a MOI of 1000 (relative gene expression = 52.5), but did not influence the type II collagen mRNA expression significantly at a MOI of 250 (5% increase ($p=0.154$). However, the relative gene expression was 8.3 (Table 2). Interestingly, the expression levels (Ct) of type II collagen in the osteoarthritic chondrocytes were higher compared with the tsT/AC62 chondrocytes (MOI=0; 43%, $p<0.01$. MOI=250; 41%, $p<0.01$. MOI=1000; 37%, $p<0.01$). But the relative gene expressions of type II collagen at the corresponding MOI were lower in osteoarthritic chondrocytes compared with tsT/AC62 chondrocytes.

A 19% ($p=0.019$) increase in aggrecan mRNA expression (Ct) and a relative gene expression of 199.9 at MOI of 1000 were detected in the AAV-TGF-β1 treated tsT/AC62 chondrocytes. AAV-TGF-β1 transduction of tsT/AC62 chondrocytes at a MOI of 250 did not lead to a significant increase in mRNA expression (6% increase ($p=0.197$)). The relative aggrecan gene expression was 8.7 in tsT/AC62 chondrocytes at a MOI of 250. The aggrecan mRNA expression (Ct) in osteoarthritic chondrocytes was

Table 2. Relative gene expressions of TGF-β1, type II collagen, aggrecan, and MMP-3

MOI of AAV-TGF-beta-1	Gene	T/AC62	*h*OC
250	TGF-beta1	13.5 ± 2.5	13.2 ± 3.5
	Type II collagen	9.3 ± 2.2	8.3 ± 2.7
	Aggrecan	8.7 ± 3.1	7.9 ± 2.7
	MMP-3	0.8 ± 0.4	0.9 ± 0.4
1000	TGF-beta1	321.6 ± 42.0	585.7 ± 59.4
	Type II collagen	113.6 ± 18.6	52.5 ± 16.8
	Aggrecan	199.9 ± 19.4	107.2 ± 16.5
	MMP-3	0.11 ± 0.03	0.19 ± 0.04

T/AC62: normal adult human articular chondrocyte cell line, hOC: human osteoarthritic chondrocytes.

increased with 19% ($p<0.01$), and the relative gene expression was 107.2 by AAV-TGF-β1 transduction at MOI of 1000. At MOI of 250 no differences were found in Ct (5% increase ($p=0.209$). The corresponding relative gene expression was 7.9. Analogous to type II collagen expression, higher expression levels (Ct) of aggrecan was found in osteoarthritic chondrocytes when compared with tsT/AC62 chondrocytes (MOI=0; 30%, $p<0.01$. MOI=250; 29%, $p<0.01$. MOI=1000; 30%, $p<0.01$), but the equivalent relative gene expressions were lower.

Both expression (Ct) and relative gene expression of MMP-3 were reduced in both tsT/AC62 chondrocytes and osteoarthritic chondrocytes when treated with AAV-TGF-β1 at MOI of 1000 (Ct = 13% ($p=0.044$); relative gene expression = 0.1, and Ct = 55% ($p<0.01$); relative gene expression = 0.2, respectively). When infecting with AAV-TGF-β1 at MOI of 250, no significant differences in Ct were found in tsT/AC62 chondrocytes or osteoarthritic chondrocytes when compared to uninfected chondrocytes. However, the relative MMP-3 gene expressions at the MOI of 250 were 0.8 in tsT/AC62 chondrocytes and 0.9 in osteoarthritic chondrocytes. In untransduced controls and at MOI of 250, higher expression levels (Ct) of MMP-3 were observed in osteoarthritic chondrocytes compared with tsT/AC62 chondrocytes (MOI=0; 44%, $p<0.01$. MOI=250; 42%, $p<0.01$). But when treating with the AAV-TGF-β1 at a MOI of 1000 no significant difference in MMP-3 mRNA expression (Ct) was found between tsT/AC62 chondrocytes and osteoarthritic chondrocytes ($p=0.07$).

Discussion

The present study shows that AAV-TGF-β1 vector transduction of human articular chondrocytes increases cellular synthesis of TGF-β1. In both primary osteoarthritic chondrocytes and a human normal articular chondrocyte cell line (tsT/AC62) this leads to increased expression of type II collagen and aggrecan, whereas the MMP-3 mRNA expression is decreased.

Traditional drug delivery methods cannot target specific joints or maintain effective concentrations of drugs for a long time. The challenge to gene-based treatment strategies is to devise methods that incorporate the correct gene or gene combination with the appropriate vector, delivered to specific target cells within the proper biological context to achieve a meaningful therapeutic response. In this perspective, the AAV vector might be an attractive tool toward the treatment and repair of damaged articular cartilage (Schwarz, 2000). AAV vectors have been demonstrated useful in transducing the three primary candidate cell types to target for genetic modification in order to treat joint diseases, which are synovial lining cells (Goater *et al.*, 2000), normal chondrocytes (Arai *et al.*, 2000; Madry *et al.*, 2003; Ulrich-Vinther *et al.*, 2002), and mesenchymal stem cells (MSCs) (Ito *et al.*, 2004). In the present *in vitro* study, the chondro-metabolic consequences of AAV-TGF-β1 gene delivery to both normal and osteoarthritic human chondrocytes were investigated. Our data demonstrates that the AAV-TGF-β1 vector efficiently transduces both osteoarthritic human chondrocytes and a human normal articular chondrocyte cell line, and additionally indicates that normal articular chondrocytes and osteoarthritic chondrocytes are equally responsive to AAV vector mediated gene transduction. We found the transduction efficiencies are consistent with previous published data on normal primary human articular chondrocytes (Ulrich-Vinther *et al.*, 2002). Madry *et al* have demonstrated that AAV-based vectors can efficiently transduce and stably express marker genes in articular chondrocytes of normal and osteoarthritic human articular cartilage (Madry *et al.*, 2003). These experiments were based on repeated *in vitro* transductions of primary chondrocytes from one patient with osteoarthritis only. However, our results, based on separate paired studies of

primary human osteoarthritic chondrocyte cultures derived from 8 patients, are backing-up the conclusion from Madry *et al.*

Furthermore, our study demonstrates the AAV-TGF-β1 transduction of normal and osteoarthitic human chondrocytes leads to elevated levels of endogenous bioactive TGF-β1. Induction of increased TGF-β1 synthesis in normal articular chondrocytes derived from various mammals have been demonstrated with other viral vector systems (Arai *et al.*, 1997; Gelse *et al.*, 2003; Shuler *et al.*, 2000; Trippel *et al.*, 2004). To our knowledge, TGF-β1 transduction of human articular chondrocytes has not yet been published.

Among the list of potentially useful cDNAs for cartilage repair are the anabolic growth factors of the TGF-b superfamily (Izumi *et al.*, 1992; Joyce *et al.*, 1990; Moses *et al.*, 1996), including TGF-β1-3 (Worster *et al.*, 2000), several of the bone morphogenetic proteins (BMPs) (Sato *et al.*, 1984; Sellers *et al.*, 1997; Wang *et al.*, 1990), insulin-like growth factor (IGF)-1 (Fortier *et al.*, 2002; Nixon *et al.*, 2001; Trippel *et al.*, 2004), fibroblast growth factors (FGFs) (Ellsworth *et al.*, 2002; Hill *et al*, 1992), and epidermal growth factor (EGF) (Osborn *et al.*, 1989). In particular, TGF-β1 has been identified as a key factor as TGF-β1 potentates expression of type II collagen and proteoglycans, enhances proliferation and differentiation, and appears to redifferentiate the phenotypically altered chondrocytes seen in osteoarthritic cartilage (Blumenfeld *et al.*, 1997; Gouttenoire *et al.,* 2004; Lafeber *et al.*, 1997; Livne *et al.*, 1994). Delivery by recombinant adenovirus of the cDNA for TGF-β1, to monolayer cultures of chondrocytes isolated from experimental animals has been shown to stimulate expression of cartilage matrix genes, resulting in increased synthesis of proteoglycan and collagen type II (Shuler *et al.*, 2000; Smith *et al.*, 2000). TGF-β1, IGF-1 and BMP-2 over-expression has also been found to rescue proteoglycan synthesis following pretreatment of the chondrocytes with IL-1, a potent inhibitor of matrix synthesis (Smith *et al.*, 2000). In concordance with these studies, the present study shows that AAV vector mediated TGF-β1 gene therapy up-regulates the anabolic metabolism in both normal and osteoarthritic articular chondrocytes *in vitro*.

Osteoarthritis is characterized by high level of degradative enzymes such as MMPs that deteriorate normal articular cartilage. The present *in vitro* experiments demonstrate that AAV-TGF-β1 transduced osteoarthritic human chondrocytes respond to the elevated endogenous production of TGF-β1 by decreasing their synthesis of MMP-3. Down-regulation of MMP-3 expression by TGF-β1 has been demonstrated by other studies as well (Shlopov *et al.*, 1999; Su *et al.*, 1996). A indirect influencing of the TGF-β1 signaling cascade *via* IL-1 signaling have been suggested (Kaiser *et al.*, 2004; Moulharat *et al.*, 2004). It is noteworthy that AAV vector mediated TGF-β1 transfer to osteoarthritic chondrocytes and the normal articular chondrocyte cell line resulted in equal increases in TGF-β1 synthesis, but that the subsequent anabolic responses to this stimulus were more pronounced in the normal chondrocytes compared with the osteoarthritic chondrocytes. It might be that primary osteoarthritic chondrocytes, which are derived from deteriorated joints characterized by chronic inflammation, have become metabolically deranged, and, thus, unable to respond sufficiently to an anabolic stimulation. Comparisons of metabolic responses the immortalized articular chondrocyte cell line may also be a part of the explanation. The hypotheses must be investigated in advanced *in vitro* experiments and *in vivo* models of osteoarthritis.

It has been shown that frequent large doses, direct intra-articular injections of TGF-β1 induce undesired effects such as synovium inflammation and osteophytes (van Beuningen *et al.*, 1994). Concerning these problems, gene transduction has an advantage by changing the promoter area of the gene, specific therapeutic gene can be expressed in a target tissue. Hence, by using a tissue specific promoter for a certain collagen, e.g. type II collagen, it would be possible to limit TGF-β1 expression on cartilage tissue and to reduce the severity of unwanted effects on other tissues. Furthermore, gene therapy approaches may be further improved by implementation of adjustable constructs such as RU486 (Hyder *et al.*, 2000), TET (Lewandoski, 2001) sensitive promoter systems.

In conclusion, the present study demonstrates that both primary osteoarthritic human chondrocytes and a human normal articular chondrocyte cell line can be genetically manipulated by a recombinant AAV vector to produce and respond to the potentially therapeutic cytokine, TGF-β1. This technology has a number of experimental and therapeutic applications, including those related to the study and treatment of arthritis and cartilage repair.

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Discussion with Reviewers

Reviewer: The authors have shown that AAV mediated gene transfer of TGF-β1 in both normal and osteoarthritic chondrocytes results in enhanced expression of TGF-β1, Type-II collagen and Aggrecan, whilst reducing the expression of MMP-3. This was performed in 2-dimensional culture plates where it is known that chondrocytes behave differently in 2D versus 3D environments; the former enhancing de-differentiation.

Do the authors have experimental data or can they speculate on the possible effect of this gene transfer in both cell types when grown in 3D constructs?

Authors: This is an important issue raised by the reviewers. *In vitro* culturing of articular chondrocytes is a difficult task. It is evident that monolayer (2-dimensional) culture conditions are not appropriate for long term studies of articular chondrocyte metabolism. In the present study, we have confirmed the metabolic maturity of the cultured chondrocytes by performing PCR-analyses on genes characterizing mature hyaline (articular) chondrocytes after eight days of culturing in monolayers (i.e. at the end of observation period). Hence, we demonstrated that de-differentiation of the articular chondrocytes did not occur with in the time span of the experiments. However, minor metabolic deterioration of the articular chondrocytes in the monolayer culture conditions metabolism can not be denied.

In previous studies (Ulrich-Vinther *et al.,* 2002; Ulrich-Vinther *et al.,* 2004), we have investigated the AAV vector transduction efficiencies in monolayer cultures of human articular chondrocyte, human articular cartilage explants, and *in vivo* models. In brief, the AAV vector mediated transduction efficiency of articular chondrocytes embedded in native 3-dimensional extra-cellular matrix decreases with the distance from cartilage surface. Thus, a decline in the number of chondrocytes, which are also expressing less trans-gene material, has been demonstrated in the remote parts of articular cartilage compared with chondrocytes located at the articular cartilage surface or at surfaces of cartilage injuries.

Novel studies investigating metabolism effects of bioactive proteins such as growth factors on articular chondrocytes cultured in three-dimensional scaffold have been initiated in our group. A synergistic effect of the three-dimensional environment and supplementation of appropriate signal proteins on hyaline cartilage genesis is hypothesized.

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