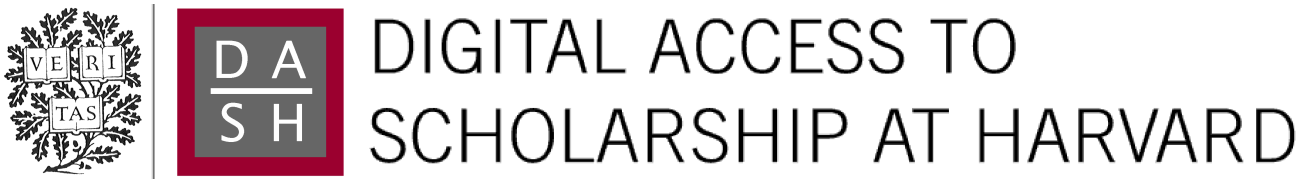

Induction of Synovial Apoptosis by Gene Transfer and Peptide Mediated Protein Transduction

Meeting abstracts

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P1

Adeno-associated virus preferentially transduces human compared to mouse synovium

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There is increasing interest in adeno-associated virus (AAV) vectors for a wide variety of gene therapy applications. AAV is a nonpathogenic human parvovirus that can mediate long-term transduction of a number of cell types without provoking a significant immune response. These properties make AAV especially attractive for use in gene therapy of rheumatoid arthritis (RA), a chronic inflammatory disease. To investigate the potential of AAV in gene therapy of arthritis, the ability of AAV to infect synovium *in vitro* and *in vivo* was tested. Three human RA synovial fibroblast cell lines and two murine (one DBA/1J and one DBA1J×C3H F1) synovial fibroblast cell lines were used to test AAV transduction *in vitro*. The cell lines (2×10^5 cells) were infected with 10^4 particles/cell of a murine IL-10-encoding vector (AAV-mIL-10) alone or with the addition of a low titer (100 particles/cell) of an E1-, E3-deleted recombinant adenovirus to provide E4orf6 activity to enhance second-strand synthesis. The supernatants were harvested from the wells at various time points and assayed for mIL-10 expression by ELISA. Both human synovial cell lines infected with AAV alone demonstrated low-level transgene expression throughout the course of the study. However, by day 10, all human cultures coinfected with adenovirus showed a 16- to 56-fold increase in mIL-10 compared to cultures infected with AAV-mIL10 alone. By day 30, a 31- to 135-fold increase was observed. No such increase was observed in any of the mouse cell lines. To determine the AAV transduction efficiency for synovium *in vivo*, human RA synovial tissues obtained from patients undergoing joint-replacement surgery were implanted subcutaneously on the backs of NOD.CB17-Prkdc SCID mice. After allowing a 2-week period for engraftment, tissues were injected with 3.4×10^{11} particles of AAV-luciferase alone or in combination with 1.0×10^{11} particles of adenovirus. Two weeks following AAV administration, the tissues were homogenized and assayed for expression of luciferase. Only the tissues coinfected with adenovirus had luciferase levels above background. A similar experiment with AAV-LacZ demonstrated X-gal staining only of synovial tissues coinfected with adenovirus. These findings demonstrate a preferential ability of AAV to transduce human, compared to mouse, synovial tissue and suggest that second strand synthesis may be a limiting factor in gene transduction. Further studies to elucidate the mechanisms limiting gene transduction in human synovium may allow optimization of this vector for the treatment of arthritis.

P2

Delivery of antisense constructs and ribozymes to inhibit cartilage destruction in the SCID mouse model of RA

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Research of the last years has demonstrated clearly the role of rheumatoid arthritis synovial fibroblasts (RA-SF) in the destruction of articular cartilage. It has been understood that RA-SF not only exhibit features of activation and altered apoptosis, but following attachment to cartilage secrete large amounts of matrix degrading enzymes that mediate the destruction of extracellular matrix. Given recent advances in the field of gene transfer, we have been working on specific strategies to interfere with the expression of disease relevant matrix degrading enzymes using the complementary approaches of ribozymes and antisense expression constructs.

Ribozymes are short RNA molecules that have catalytic activity and are capable of cleaving mRNA thus inhibiting its translation. We have used retroviral gene transfer of ribozymes against MMP-1 as well as cathepsins B and L to inhibit the expression of these enzymes in RA-SF both *in vitro* and when implanted together with normal articular cartilage into severe combined immunodeficient (SCID) mice. As demonstrated *in vitro*, gene transfer of such ribozymes results in a sustained, up to 60% decrease of enzyme production in RA-SF over 60 days. Currently, SCID mouse experiments are underway to study the effect of gene transfer with these ribozymes on cartilage degradation *in vivo*.

To evaluate the potential of antisense constructs as an alternative approach, we have generated antisense constructs against the novel MT1-MMP and transduced RA-SF using a retroviral system. First data indicate a high efficacy of MT1-MMP antisense in inhibiting the production of MT1-MMP. However, constantly high levels

of MT1-MMP antisense RNA are needed for a sustained effect in RA-SF. The focus of our current efforts is to optimize the construct both in terms of specificity and its level of expression.

P3

Clinical evaluation of autologous chondrocytes for joint repair

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Autologous cultured chondrocyte transplantation was introduced in Sweden in 1987 for the treatment of full thickness chondral defects of the knee. The results from the first 219 patients with a follow up of 2–10 years treated using this technique are reported. Patients were assessed with three types of endpoints: patient and physician derived clinical rating scales, arthroscopy assessments of cartilage fill, integration, and surface hardness and histology of graft biopsies. There was a high percentage of good to excellent results (84–90%) in patients with different types of single femoral condyle lesions, while other types of lesions had a lower degree of success (mean 74%). Furthermore, the long-term durability of the grafted area has been evaluated in a group of 61 patients with femoral condyle and patellar lesions who where followed for a mean of 7.4 (range 5–11) years and where the durability was calculated as the comparison between the long-term follow up and clinical status at 2 years post surgery. At 2 years, 50 of 61 patients treated were classified as satisfactory and at mean 7.4 years follow up, 51 of 61 were graded satisfactory. Twelve defects tested biomechanically demonstrated stiffness values ≥90% of normal in 67% of the patients and 8/12 graft biopsies showed hyaline-like characteristics. The good clinical long-term outcomes of autologous chondrocyte transplantation in these studies are encouraging. Future research programs contain a series of randomized studies on the different clinical repair methods available and more research on the cell sources, matrices and repair tissue evaluation.

P4

Viral gene transfer for bone healing

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Objectives: Local gene transfer to bone and adjacent tissues offers promise in cases of bone healing disorders such as segmental bone defects, non-unions, and aseptic loosening of endoprotheses.
Method and results: Studies in which osteoinductive genes have been administered therapeutically are predominantly segmental defect models in rabbits and rats. One additional study has been performed to prevent bone loss in mice.
The administration of different marker genes to segmental defects induced transient gene expression locally for up to 6 weeks. β-galactosidase expression was seen after injection of adenoviral vectors encoding the LacZ gene within the callus tissue, the bony ends adjacent to the cuts, and the surrounding muscle. After injection of Ad-luciferase gene expression was also found predominantly locally, and besides that very low expression in the liver for up to 5 days, whereas local expression within bone lasted up to 6, within the surrounding soft tissues up to 3 weeks. No transgene expression was seen in the contralateral limb, lung, or spleen.
Injection of adenoviral vectors carrying BMP-2 cDNA led to healing of the segmental defects after 8–12 weeks, the untreated control defects did not heal. Vectors encoding the transforming growth factor-β1 gene increased matrix formation within the defects, but resulted not in complete mineralization of the newly formed callus as seen after transduction with the BMP-2 cDNA. The results were judged by radiographic, histologic, histomorphologic, and biomechanical criteria.
Conclusion: The data encourage the further development of genetic approaches to enhancing bone formation in cases of bone disorders.

P5

Osteoprotegerin (OPG) gene therapy in animal models of osteoarticular disease

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The soluble decoy receptor osteoprotegerin (OPG) regulates bone resorption by inhibiting osteoclast formation, function and survival. We investigated OPG gene therapy as a means of ameliorating chronic osteoarticular disease using a mouse ovariectomy (OVX) model of estrogen deficiency-induced osteoporosis (*Mol Ther* 2001, **3**:1–9). Young adult female mice injected once with an adenoviral (Ad) vector carrying a human fusion protein combining the OPG ligand-binding and immunoglobulin constant domains (hOPG-Fc) developed serum OPG levels that exceeded the threshold needed for efficacy (as assessed by a marked increase in bone density) for over 12 months. The extent of OPG production and skeletal enhancement was titer-dependent. Mice subjected to OVX or sham surgery and then treated with Ad-hOPG-Fc had significantly more bone volume and fewer osteoclasts in axial and appendicular bones after 4 weeks. In contrast, animals given OVX and either a control vector expressing β-galactosidase or vehicle had significantly less bone than did comparably treated, sham-operated mice. This study confirms that a single Ad gene transfer can produce persistent high-level OPG expression and shows that OPG gene therapy may prove useful in treating osteoporosis. We anticipate that OPG gene therapy will offer similar promise as a bone-sparing agent in chronic arthritis since we have shown previously that injected OPG significantly inhibits skeletal erosion in the Lewis rat model of mycobacterial-induced adjuvant arthritis (*Nature* 1999, **402**:304–309).

P6

In vivo selection of synovial specific targets using phage display technology

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Phage display technology has been demonstrated to be a powerful tool in identifying peptide motifs critical for cell adhesion [1]. We aim to use this technology to identify novel synovial determinants using the human synovium/SCID mouse transplantation model.
First, to validate the capacity of the library to identify specific ligands, we tested *in vitro* its ability to recognize the integrin $\alpha_v\beta_3$ which displays a specific RGD peptide binding motif [2]. Three rounds of selection were performed on a BSA blocked, $\alpha_v\beta_3$ (0.5 μg/well) coated microtiter plate. Bound phages were recovered by acid elution and amplified in *E coli*. As expected, a striking enrichment in the third round of selection was obtained for RGD containing clones. In addition, novel flanking sequences were identified in six of the clones.

Having validated the library, to select *in vivo* synovial determinants, three rounds of enrichment were performed using 6-week-old SCID mice transplanted with human synovium [3]. Four weeks post-transplantation, 10^9 TU/ml phages were injected intravenously into the tail vein. The phages were allowed to recirculate for 5 min and animal sacrificed following perfusion through the heart to remove unbound phages. In each round a significant enrichment for synovial tissue was obtained. We are currently engaged in the characterization of these novel determinants.

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P7

Evidence for re-differentiation of human chondrocytes seeded on a hyaluronan derivative scaffold

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Association of biomaterials with autologous chondrocytes promises to provide a new generation of implantable devices for cartilage repair. HYAFF®-11 is a recently developed hyaluronic-acid based biodegradable polymer, that has been shown to provide successful cell scaffold for tissue-engineered repair. The aim of this study was to estimate the capacity of HYAFF®-11 to support the growth of human chondrocytes and their original phenotype. To this end, human chondrocytes (10^6 cells/cm^2) were seeded on HYAFF®-11 and their proliferation and differentiation were assessed at different time points. Gene expression for collagen I, II and aggrecan was revealed by RT-PCR evaluating the presence of the specific mRNAs, while histochemical analyses for these proteins were performed by the use of specific monoclonal antibodies. Our data indicate that human chondrocytes seeded on HYAFF®-11 after expansion *in vitro*, re-express and produce collagen type II and aggrecan and downregulate the production of collagen type I.

HYAFF®-11 was also utilized to stabilize the phenotype of an immortalized human chondrocyte line obtained by liposome-mediated transfection with the p16HHMo plasmid encoding two HPV16 early function genes.

These results prove that normal and transfected human chondrocytes can grow and differentiate on a hyaluronan-based scaffold (HYAFF®-11) which can be used as a delivery vehicle for the repair of articular cartilage defects.

P8

AKT regulates TNF-α-mediated apoptosis of rheumatoid arthritis synovial fibroblasts

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Objective: To determine whether TNF-α driven proliferation of rheumatoid synovial fibroblasts is associated with upregulation of the serine/threonine kinase B (PKB)/AKT activity and RA synovial fibroblast survival.

Method: Staining of phosphorylated AKT was done using antiphosphorylated Thr308-AKT antibody. Phosphorylated AKT was analyzed by Western blot, and AKT activity was analyzed using a kinase assay. The cytotoxicity of TNF-α treatment or TNF-α plus AKT activity inhibitor wortmannin, TNF-α plus dominant mutant (AdAkt-DN) or AdPTEN was analyzed using ATPLite assay.

Results: The levels of phosphorylated-Akt are higher in RASF than in OASF, as demonstrated by immunohistochemical staining, immunoblot analysis and an Akt kinase assay. The levels of phosphorylated Akt and Akt kinase activity were increased by stimulation of primary RASF with TNF-α (10 ng/ml). Treatment of RASF with the PI 3-kinase inhibitor, wortmannin (50 nM), plus TNF-α resulted in apoptosis of 75 ± 8% of RASF within 24 h. This proapoptosis effect was specific for Akt, as equivalent levels of apoptosis were observed upon TNF-α treatment of RASF transfected with adenovirus expressing a dominant negative-Akt (AdAkt-DN) and with an adenovirus expressing PTEN (AdPTEN), which opposes the action of Akt.

Conclusion: These results indicate that phosphorylated Akt acts as a survival signal in RASF and contributes to the stimulatory effect of TNF-α on these cells by inhibiting the apoptosis response. This effect was not observed in OSAF, and may reflect the pathophysiologic changes associated with the proliferating synovium in rheumatoid arthritis.

P9

AAV mediated delivery of IL-4 prevents collagen-induced arthritis

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Immunomodulation of autoimmune inflammatory diseases like rheumatoid arthritis can be achieved by anti-inflammatory T2 cytokines such as interleukin (IL)-4 administered by gene therapy. Engineered cells secreting cytokines was the first demonstrated efficient gene therapy in animals models of arthritis. Adeno-associated virus (AAV) are new gene therapy vectors exhibiting a number of advantages over the others vectors. AAV is a small DNA virus of the Parvoviridae family. In human populations, the overwhelming majority of individuals have been exposed to the AAV, which doesn't seem to have known pathological effects. In addition to being safe, AAV vectors can infect a broad spectrum of host cells, at any phase of their division cycle. Furthermore, recombinant AAV vectors contain no viral genes but only the transgene flanked by two inverting terminal repeats (ITRs), which could package moderately sized transgenes, such as those encoding for cytokines.

We recently investigated the efficiency of adeno-associated virus (AAV) vectors in collagen-induced arthritis (CIA). After injection of AAV-lacZ in the tarsus area of mice, the expression of the transgene was localized in the deep muscle cells near the bone. LacZ expression was found in liver, heart and lung after intramuscular injection of AAV-LacZ, showing a spread of the vector over the body. Anti-AAV neutralizing antibodies were detected in the serum after intramuscular injection of AAV-LacZ, but they didn't alter the transgene expression after re-administration of AAV-LacZ. Long-term IL-4 expression persisted 129 days after intramuscular injection of 3.7×10^{10} or 11.2×10^{10} AAV-IL-4 pp (average 7.7 or 17.5 pgIL-4/mg proteins, respectively). More importantly, the treatment of CIA with AAV-IL-4 vector in mice produced a therapeutic benefit, since we show a diminished prevalence of the disease, a significant reduction in paw swelling,

attenuated histological synovitis and a 10 day delayed onset of arthritis. These data show evidence that AAV vector-mediated gene therapy using a T2 cytokine is efficient in an animal model of rheumatoid arthritis.

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P10

IKKβ and p53 as therapeutic targets for arthritis

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The disparity between the proportion of cells with DNA strand breaks and the low number of apoptotic cells in rheumatoid synovial tissue suggests impaired apoptosis. Therefore, therapeutic strategies aimed at induction of apoptosis in rheumatoid synovial tissue may be attractive. Adjuvant arthritis (AA) in rats is among the most commonly used model for rheumatoid arthritis (RA). To characterize the extent to which apoptosis occurs in the natural course of the disease, we evaluated the number of apoptotic cells and the expression of p53 in various phases of AA. We found that significant apoptosis only occurs late in AA and this is concordant with marked p53 overexpression, making it a useful model for testing proapoptotic therapies. Intra-articular adenoviral gene transfer of wild-type p53 resulted in an incremental increase in p53 expression in the injected joints, but this therapy did not have a beneficial clinical effect, since there was already dramatic p53 overexpression in the later stages of AA.

NF-κB activation is another factor involved in the persistent activation of synovial cells and impaired apoptosis. IκB kinase β (IKKβ) is a key regulator of NF-κB. We observed the development of arthritis after intra-articular adenoviral gene transfer of IKKβ-wt into the joints of normal rats. Increased IKK activity was detectable in the Ad.IKKβ-wt injected ankle joints, coincident with enhanced NF-κB binding activity. Conversely, intra-articular gene transfer of Ad.IKKβ-dn significantly ameliorated the severity of adjuvant arthritis in Lewis rats. This effect was accompanied by a significant decrease in levels of NF-κB binding activity. Targeting IKK activity may represent a valid new strategy for the treatment of RA.

P11

Engineering T cells and molecules for targeting joints and inflammation

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Objective: Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterised by cartilage and bone erosion. In order to target the joint and the inflammation process specifically, avoiding systemic secondary effects of biological compounds, we have: engineered T cells to recognise collagen type II (CII), a main constituent of cartilage, with a chimeric receptor composed of a scFv extracellular domain which binds to CII and an intracellular domain which signals inducing cytokine production and T cell proliferation; and modified cytokines with a biodegradable 'shell' that is removed in sites of inflammation by MMP activity.

Results: Primary T cells grafted with the chimeric scFv receptor with a ζ chain intracellular domain via retroviral transduction, form homodimeric receptors in the cell surface recognising CII *in vitro* both in solution and attached to plastic and respond producing IL-2 and IFN-γ. KLH-specific T cells produce also IL-4 when challenged with KLH but upon grafting with the chimeric receptor lose the ability to produce this Th2 cytokine. This is in agreement with preliminary findings that engineered T cells are arthritogenic *in vivo* following CII challenge.

We have produced a fusion protein between a cytokine and a protective protein subunit linked via an MMP cleavage site. We show *in vitro* that the engineered fusion protein is inactive (incapable of binding to its cellular receptors) unless cleaved by MMP1, MMP3 or by synovial fluid of RA patients. Intramuscular injection of the plasmid expressing a 'mutated' active versus the 'inactive' form of the protein showed that the latter was more effective as expected in the treatment of collagen-induced arthritis in DBA/1 mice.

Conclusion: Engineering cells and molecules to achieve site-specific activation has advantages for gene therapy of RA.

P12

Elimination of rheumatoid synovium *in situ* by Fas ligand 'gene scalpel™'

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Introduction: Surgical synovectomy to remove the inflammatory synovium, can temporarily ameliorate rheumatoid inflammation and delay the progress of joint destruction. An efficient medically induced programmed cell death (apoptosis) in the RA synovium might play a similar role as synovectomy. Gene transfer of FasL has increased the frequency of apoptotic cells in collagen induced mouse arthritis synovium. In this study, we investigated whether a repeated FasL gene transfer could function as a molecular synovectomy to remove human inflammatory synovial tissue *in situ*.

Method: RA synovium and cartilage from joint replacement surgeries of 5 patients were cut into small pieces at about $2 \times 3 \times 3\,mm^3$/piece and then was grafted subcutaneously into male C.B-17 SCID mice aged 6–7 weeks at 0.2 g tissue per mouse. Injections of a recombinant FasL adenovirus into the grafted synovial tissue at the dosage of 10^{11} particles per mouse were performed twice/month. The control groups were treated with recombinant LacZ adenovirus at the same dosage and time points as Ad-FasL administration.

Results: Compared to the control Ad-LacZ injected RA synovium, the Ad-FasL injected RA-synovium was dramatically reduced in size and weight. After 8 weeks the weights of grafted RA synovium were 0.039 ± 0.024 g in the Ad-FasL treated group and 0.152 ± 0.021 g in the Ad-LacZ treated group ($P < 0.01$). Both synoviocytes and mononuclear cells were greatly decreased after the 2 months treatment with Ad-FasL.

Conclusion: Our *in vivo* investigation of gene transfer to human synovium in SCID mice suggests that arresting inflammatory synovium at an early stage of RA by intra-articular gene transfer of an apoptosis inducer, such as FasL, might be possible.

P13

Double and triple gene transfer in arthritis

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Objective: As single gene transfer using virus-encoded inhibitory molecules such as IL-1Ra, IL-10 and sTNFαRP55 resulted in different benefical effects on cartilage destruction in the SCID mouse model for RA, we examined the outcome of double and triple gene transfer in this model and analyzed the molecular effects induced by this approach.
Method: RA synovial fibroblasts were transduced using adenoviral sTNFαRp55 and retroviral IL10- or IL1Ra-encoding MFG vectors. Thereafter, transduced cells were mixed in various double and triple combinations and coimplanted with human cartilage for 60 days in SCID mice. Protein expression of the transduced genes was monitored throughout the experiment. RNA of transduced cells was isolated and expression of proto-oncogenes and signaling molecules before and after gene transfer was analyzed using RNA arbitrarily primed PCR (RAP-PCR), cDNA expression array and real-time PCR.
Results: When compared to single gene transfer, combination of IL-1Ra and IL-10 overexpression showed the strongest additive effect resulting in both decrease of perichondrocytic cartilage degradation and reduction of invasion of fibroblasts into the cartilage. IL-1Ra and IL-10 double gene transfer resulted also in distinct alterations of gene expression of molecules involved in cell activation and metabolism. In contrast, double gene transfer with TNFαRp55 did not improve the beneficial effects of IL-10 or those of IL-1Ra. Moreover, triple gene transfer using these three inhibitory molecules partly antagonized the benficial effects observed in double gene transfer both in the SCID mouse as well as on the molecular level.
Conclusion: In summary, double gene transfer of inhibitory molecules results in additive effects on cartilage protection and expression of genes involved in cartilage degradation in RA, whereas a less beneficial outcome may result following gene transfer of additional genes.

P14

Bone stem cell mediated gene therapy and tissue engineering

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Mesenchymal stem cells (MSCs) are key regulators in senile osteoporosis and they are suitable candidates for stem cell mediated gene therapy in local and systemic bone disorders and tissue engineering. We hypothesized that genetically engineered MSCs, expressing rhBMP2, can be utilized for gene therapy targeted to bone regeneration and bone/cartilage tissue engineering. We conditionally expressed rhBMP2 in mouse and human mesenchymal stem cells. RhBMP2 expressing clones (tet-off), AAV-BMP2 and adeno-BMP2 spontaneously differentiated into osteogenic cells *in vitro* and *in vivo*. Clones were transplanted and tracked *in vivo* in radial segmental defects (regenerating site) and in ectopic muscular and subcutaneous sites (nonregenerating sites). *In vitro* and *in vivo* analysis revealed rhBMP2 expression and function, confirmed by PCR, RT-PCR, ELISA, Westerns, immunohistochemistry, and bioassays. Secretion of rhBMP2 *in vitro* was controlled by tetracycline (in the designated clone) and resulted in secretion of 1231 ng/24 h/10^6 cells. Quantitative Micro QCT three-dimensional reconstruction revealed complete bone regeneration regulated by tetracycline *in vivo*, indicating the potential of this platform for bone and cartilage tissue engineering. Moreover, transplants of rhBMP2 expressing clones (tet-off) exhibited more than a 10-fold increase in angiogenesis, a crucial element in tissue engineering. We are currently characterizing a novel tissue engineering platform composed of engineered cells and scaffolds, creating 3D bone tissue in bioreactors, clearly indicating that our regulated clones and polymeric scaffolds can be utilized for tissue engineering. Our study should lead to the creation of gene therapies for systemic and local bone diseases in humans and bone/cartilage tissue engineering.

P15

Enhanced chondrocyte apoptosis leads to abnormal cartilage in rats and cats with glycosaminoglycan storage diseases

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The mucopolysaccharidoses (MPS) are a family of glycosaminoglycan (GAG) storage diseases caused by the deficient activities of specific lysosomal enzymes. A major feature of the MPS disorders is abnormal cartilage and bone development leading to short stature, dysostosis multiplex, and degenerative joint disease. Ultrastructurally, the cytoplasm of affected MPS chondrocytes is filled with membrane bound vacuoles containing undegraded GAGs. Clusters of hypertrophic chondrocytes form in these disorders, leading to the disruption of the normal cartilage organization. To further investigate the cellular pathology of the MPS disorders, primary cultured chondrocytes and articular cartilage were examined from cats and rats with MPS type VI (Maroteaux–Lamy disease). Markedly increased numbers of apoptotic chondrocytes were identified by TUNEL staining and immunohistochemically with anti-poly (ADP-ribose) polymerase antibodies in the MPS animals as compared to age-matched controls. The prevalence of apoptotic cells was correlated with increased levels of nitrite and tumor necrosis factor-α. Marked proteoglycan depletion also was observed in the MPS epiphyses by Safranin-O staining, and the presence of excess collagen type II was detected by immunostaining. A model of MPS endochondryl bone pathology is proposed in which an inflammatory response to mechanical trauma is caused by the deformed and irregular subchondral bone contours, leading to increased nitric oxide (NO) production in response to TNF-α, thereby inducing chondrocyte cell death. Novel treatment strategies for these disorders might, therefore, be considered to prevent inflammation or inhibit NO production as an alternative to providing the missing enzymatic activities.

P16

Transfer of protease inhibitors to inhibit cartilage destruction

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Objective: Proteases such as serine proteases and matrix metalloproteases (MMPs) are involved in cartilage destruction in rheumatoid arthritis. The effects of gene transfer of plasmin inhibitors and tissue inhibitors of metalloproteinases (TIMPs) on cartilage degradation and invasion by rheumatoid synovial fibroblasts were investigated.
Method: Replication defective adenoviral vectors were used for transduction. Genes encoding the following inhibitors were used: the plasmin inhibitor, bovine pancreatic trypsin inhibitor (BPTI); a cell surface-targeted plasmin inhibitor, ATF.BPTI, a hybrid protein of BPTI and a ligand of the cell surface uPA-receptor, ATF; TIMP-1; and TIMP-3. Cartilage degradation was investigated in an *in vitro* model using a radiolabeled cartilage-like matrix. The invasive behavior of rheumatoid synovial fibroblasts was studied *in vitro* in a Transwell model and *in vivo* in the SCID mouse co-implantation model.
Results and conclusion: Cartilage degradation was significantly reduced by gene transfer of BPTI and ATF.BPTI. The effect of ATF.BPTI was significantly stronger than that of BPTI suggesting that targeting protease inhibition to the cell surface improves the inhibitory effect. Gene transfer of ATF.BPTI, TIMP-1 and TIMP-3 significantly inhibited cartilage invasion. These results indicate that cartilage degradation and invasion can be inhibited by gene transfer of inhibitors of plasmin and MMPs. Inhibition of proteases at the site of joint destruction through gene transfer may provide a novel therapeutic strategy to limit the progression of joint destruction in rheumatoid arthritis.

P17

Local interleukin-12 gene transfer promotes conversion of an acute to a chronic destructive murine streptococcal cell wall arthritis

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Objective: Interleukin-12 is a pleiotropic cytokine that is produced by mononuclear phagocytes, dendritic cells and B cells, and it promotes the growth of activated T cells and NK cells. IL-12 selectively generates the development of naïve T cells into Th1 cells. IL-12 is effectively produced by macrophages upon stimulation with LPS, several bacteria and intracellular parasites. The goal of the present was to determine whether local overexpression of IL-12 converts an acute joint inflammation to a chronic destructive arthritis.
Method: SCW arthritis was induced in IL-12 deficient mice to examine the IL-12 dependency. C57Black/6 mice were injected intra-articularly with either saline, 10^7 pfu control vector (Ad5del70-3) or IL-12 vector (AdmIL-12.1) into the right knee joint one day before the mice were injected intra-articularly with 25 µg SCW fragments. Thereafter, joint swelling, chondrocyte proteoglycan (PG) synthesis and joint destruction were examined. In addition MMP activity was visualized by VDIPEN staining. Arthritis was exacerbated by intravenous injection of 100 µg SCW fragments.
Results: IL-12-deficient mice showed reduced joint swelling after injection of SCW fragments. High levels of IL-12 (up to 20 ng/ml at day 1) could be detected after application of AdIL-12 vector. After 14 days still expression of IL-12 (1 ng/ml) was found locally without significant inflammation. Local expression of IL-12 reveals to aggravate SCW arthritis as determined by enhanced joint swelling and inhibition of chondrocyte PG synthesis. Histology taken at day 21 showed a chronic inflammatory process in the AdIL-12 transfected knee joints. Enhanced cartilage PG depletion and cartilage destruction was noted in the AdIL-12 group, whereas this was not seen in the Ad5del70-5 group. In line with these findings, metalloproteinase activity, visualized by VDIPEN expression in the cartilage layers, was only found in AdIL-12 group. In addition, systemic challenge of arthritis was only possible in the AdmIL-12 group, indicating a T cell mediated process.
Conclusion: These results indicate that local expression of IL-12 can promote conversion of an acute to a chronic destructive arthritic process.

P18

inflammation-inducible intra-articular production of human IL-1 receptor antagonist results in a more efficient inhibition of collagen-induced arthritis than does constitutive expression of the same transgene

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Objective: To achieve disease-inducible expression of recombinant anti-inflammatory proteins in order to allow autoregulation of drug dose by natural homeostatic mechanisms.
Method: We compared a disease-inducible, two component expression system (C3-Tat/HIV) with the constitutive cytomegalovirus (ie CMV) promoter in the polyarticular collagen-induced arthritis (CIA) model. DBA/I mice were immunized with bovine type II collagen and boostered on day 22. On day 22, mice without any clinical signs of arthritis were selected and 10^e7 PFU of the adenoviral vectors (Ad5.CMV-Luc, Ad5.CMV-IL-1Ra, or Ad5.C3-Tat/HIV-IL-1Ra) that contained either luciferase (Luc) or the human IL-1Ra gene under control of one of the two promoters were used to transfect the synovial lining of both knees. The injected knee joints and ipsilateral paws were then scored for signs of arthritis and at the end histology was taken.
Results: Inducible promoter-driven IL-1Ra expression resulted in significantly improved inhibition of CIA than did CMV-driven IL-1Ra production. Moreover, overexpression of IL-1Ra in the knee joints also prevented CIA in the ipsilateral paws.
Conclusion: Our data demonstrate the feasibility of an inducible expression system for producing a transgene for treatment of arthritis; and show that this system is more effective than strong, constitutive transgene expression for preventing collagen-induced arthritis in mice.
Acknowledgement: This study was financially supported by the Dutch Arthritis Association (941) and the Dutch Organization for Scientific Research (902-27-218).

P19

Using a tropism-modified adenoviral vector for the intra-articular production of IL-1 receptor antagonist results in a more efficient inhibition of collagen-induced arthritis than does a conventional Ad5 vector

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Objective: Introducing an Arg-Gly-Asp (RGD) motif in the HI loop of the fiber knob results in the ability of the adenovirus to utilize the αvβ3 and αvβ5 integrins instead of the coxsackie-adenovirus receptor for cell recognition. In this study we compared the transfection efficiency and the effect of intra-articular IL-1Ra gene delivery on collagen-induced arthritis (CIA) using RGD modified and conventional Ad5 vectors.
Method: DBA/1 mice were immunized with collagen type II, and boostered 22 days later. Four days after the booster, 10^7 pfu of the Ad5Luc, Ad5Luc.RGD, Ad5mIL-1Ra, or Ad5mIL-1Ra.RGD were injected bilaterally into the knee joint cavity. The clinical score (scale 0–2) of the ipsilateral paws was taken during the course of arthritis, and the knees were scored (scale of 0–3) at the end of the experiment.
Results: 24-h after the intra-articular injection into normal knee joints the luciferase (Luc) production was around 22 times higher using the Ad5Luc.RGD as compared to a conventional Ad5Luc vector. Intra-articular injection of 10^7 pfu adenoviral vectors at day 26 of immunization resulted in a significant inhibition of CIA in the Ad5mIL-1Ra.RGD group (clinical score of the knee at day 38 was 0.82 ± 0.24) as compared to the other groups (clinical scores were 1.57 ± 0.24 for the Ad5mIL-1Ra group and 1.86 ± 0.21 for the Ad5Luc.RGD group).
Conclusion: Our data demonstrate that the RGD modification of adenoviruses improved the synovial transfection efficiency; and that the Ad5IL-1Ra.RGD vector was more efficacious in the treatment of arthritis than the conventional vector with the same transgene.
Acknowledgement: This study was financially supported by the Dutch Arthritis Association (941) and the Dutch Organization for Scientific Research (902-27-218).

P20

Local IL-17 gene therapy accelerates collagen arthritis with severe bone erosion and rank ligand and rank expression in synovial infiltrate and at bone erosion sites

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Objective: To examine the effects of local IL-17 application in the knee joint of type II collagen immunized mice on the induction of bone erosion.
Method: Collagen-induced arthritis (CIA) was induced in male DBA-1 mice by immunizing intradermally at the base of the tail with suboptimal dose of bovine type II collagen. On day 21, mice were given an intraperitoneal booster injection of the same dose of type II collagen dissolved in PBS. Just before expected onset, mice were intra-articularly injected into the right knee joint with 10^7 pfu of either an IL-17 expressing (AdIL-17) or control (AdControl) recombinant human type 5 adenovirus vector. Five days after the intra-articular injection of the viral vector arthritis was monitored visually and joint pathology was examined by histology. Formation of osteoclast-like cells was determined by tartrate-resistant acid phosphatase (TRAP) staining. In addition, RANKL and RANK protein expression was evaluated by specific immunohistochemistry.
Results: Local IL-17 over-expression in the knee joint of type II collagen immunized mice promotes synovial inflammation. Five days after viral injection of AdIL-17 histologic analysis showed aggravation of bone erosion in the patella and femur/tibia region compared with the control vector group. Induction of bone destruction by IL-17 was accompanied with marked tartrate-resistant acid phosphatase (TRAP) activity in the bone marrow and at bone erosion sites, indicating that IL-17 accelerates the formation of osteoclast-like cells. Interestingly, local IL-17 promotes local protein expression of RANKL and its receptor RANK in the synovial infiltrate and at bone erosion sites compared with the control vector group.
Conclusion: These data show that local IL-17 gene therapy during onset of collagen arthritis promotes osteoclastic bone erosion accompanied with accelerated expression of local RANKL and its receptor RANK. These findings suggest IL-17 to be a potent stimulator of osteoclastogenesis during arthritis.

P21

Update on the contralateral effect in rabbit and mouse models

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While screening several anti-inflammatory gene products for efficacy in rabbit and murine models of RA by intra-articular adenoviral injection, a therapeutic effect was observed in both the treated and untreated contralateral joints. This contralateral effect was observed following adenoviral gene transfer of IL-1Ra, sIL-1 receptor, sTNF-receptor, vIL-10 and IL-4. Several different experiments suggested that the therapeutic effect may be due, in part, to the migration of a population of virally transduced antigen presenting cells (APC) from the treated joint to the regional lymph nodes and the untreated arthritic joints. Consistent with this model, we have demonstrated that direct intravenous injection of genetically modified DC expressing IL-4, FasL, IL-1Ra or CTLA4-Ig resulted in suppression or elimination of established collagen induced arthritis. However, intra-articular *ex vivo* delivery of retrovirus transduced syngeneic synoviocytes expressing anti-inflammatory factors conferred a similar therapeutic effect in treated as well as untreated contralateral joints. The results from our *ex vivo* and *in vivo* studies suggested that trafficking of vector-modified inflammatory cells may not be the main mechanism responsible for the observed contralateral effect. We now propose that local, intra-articular expression of anti-inflammatory factors may be able to modify the function of APCs that are then able to modulate the immune response in the contralateral joint. In support of this model, injection of APC treated with either recombinant IL-10 protein or infected with Ad.vIL-10 were able to block the DTH response in both the treated and untreated paws. We also have demonstrated that exosomes derived from Ad.vIL-10 infected APC were able to reduce the DTH response in both the treated and untreated paws. Taken together these results suggest that modified APC as well as APC derived-exosomes can confer a novel protective, anti-inflammatory effect in treated and untreated contralateral joints.

P22

Viral, nonviral and peptide-mediated intra-articular transfer of genes and proteins

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We have been examining the ability of different vectors to transfer therapeutic genes directly to joints following intra-articular injection. To date, we have examined the efficiency of intra-articular gene transfer to the rabbit knee mediated by adenoviral, adeno-associated virus (AAV), retrovirus and herpes simplex virus vectors as well as by over 100 different nonviral formulations. Moreover, we have examined the ability to repeat dose with the different types of viral vectors. A summary of our experiments to identify a clinically useful vector for intra-articular gene transfer will be presented. In an attempt to improve intra-articular gene transfer, we also have screened an M13 peptide phage display library for peptides able to facilitate internalization into synovial cells. We have identified a class of cationic peptides (PTD), similar to the cationic protein transduction domains found in Antennapedia (Antp) and HIV TAT peptide, as well as a class of more neutral and hydrophobic peptides (HAP). To determine the ability of the PTD and HAP peptides to facilitate internalization, the peptides were biotinylated and coupled to avidin-β-gal or streptavidin-Cy3. Although internalization mediated by the HAP peptides in culture and *in vivo* was not as efficient as the cationic peptides, they appeared to be more cell type specific. Several of the HAP and PTD peptides were fused to an antimicrobial peptide, KLAK, generating novel apoptotic peptides. These fusion peptides were able to impair rabbit and human synovial cell viability through disruption of mitochondria and induction of apoptosis. Intra-articular injection of these apoptotic peptides into arthritic rabbit joints resulted in extensive synovial cell apoptosis as well as reduction in leukocytic infiltration. An update of our progress using the different internalizing peptides for delivery of therapeutic proteins and genes will be presented.

P23

In vitro and *in vivo* differentiation of mesenchymal stem cells into chondrocytes

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Mesenchymal stem cells are multipotent cells that have the potential to differentiate toward multiple lineages, such as adipocytes, myocytes, osteocytes and chondrocytes. Both primary stem cells originating from bone marrow and immortalised cell lines retain their differentiation capacities when cultured *in vitro* under specific conditions. Growth factors of the TFG-β superfamily have been shown to have the properties of inducing *in vitro* osteogenic and chondrogenic differentiation. The purpose of this work was to compare the potential of different growth factors (BMP-2, CDMP-1 and CDMP-2) and the Sox-9 chondrocyte-specific transcription factor to induce the chondrogenic differentiation of mesenchymal stem cells both *in vitro* and *in vivo*.

To this aim, we first derived mesenchymal stem cells (from the murine C3H10T1/2 line) constitutively expressing either one of the growth or transcription factor. The chondrogenic potential of these factors was then assessed *in vitro* using the micropellet culture system both by RT-PCR and immunohistochemistry. Those cells were then embedded in a type I collagen matrix and subcutaneously implanted in SCID mice. The matrices were retrieved after 20 days and processed for routine histology and immunohistochemistry analysis. Parallely, BMP-2-expressing stem cells were injected into the intra-articular space of SCID mouse knee joints to determine their capacities of *in situ* differentiation and cartilaginous matrix formation.

Intra-articular injection of mesenchymal stem cells engineered to express BMP-2 gave rise to the formation of bone in the knee joint and at extra-articular sites, particularly in muscle. The need for differentiation factors, specific for cartilage, is underlined.

P24

Inhibition of collagen-induced arthritis in mice by inducible AAV-mediated transfer of viral IL-10 gene

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Objective: The purpose of this study is to assess the therapeutic efficiency of vIL-10 gene transfer mediated by a tetracycline-inducible AAV vector in mice model of arthritis.

Method: The AAV-tetON-vIL10 vector (1.5×10^9 pi) was injected intramuscularly 3 weeks before immunization with bovine type II collagen. DBA1 mice with collagen-induced arthritis then received dox diet (200 mg/kg) from days 21–42 of immunization. Severity of arthritis was determined by measuring paw swelling, evaluating X-ray and histological paw features. Expression of vIL-10 was measured by RT-PCR and ELISA in injected muscle.

Results: The incidence and severity of arthritis was significantly reduced at macroscopic, radiologic and histologic levels in the group of animals treated with AAV-TetON-vIL10 vector plus doxycycline, compared with vector alone or control groups (AAV-GFP + doxycycline and doxycycline alone). Only 30% of mice developed arthritis against 89% in controls. Mice from the control groups showed earlier onset of arthritis, with clinical signs of the disease starting on day 32.63 ± 4.07, compared with 36.67 ± 3.51 in the AAV-TetON-vIL10 injected group treated with doxycycline ($P = 0.033$). The benefit was not only observed at the inflammatory level, but also on cartilage and bone erosion ($P < 0.01$). Gene transfer led to detectable levels of vIL-10 transcript and protein in the AAV-tetON-vIL10 injected muscles, inducible by doxycycline. A basal transcription of vIL-10 was observed, significantly induced by doxycycline ($\times 2.7$, $P = 0.0094$), leading to a higher secretion of vIL-10 protein (284.4 ± 358.2 versus 181.9 ± 130.2 pg/mg protein, NS). The biodistribution of the vector genome beyond the site of injection was determined by RT-PCR, vIL-10 transcript was found in five out of 29 spleens.

Conclusion: These data support AAV-tetON-vIL10 as a valuable approach for anti-inflammatory gene therapy in RA.

P25

Development of lentiviral vectors for gene therapy of arthritis

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The delivery to joints of genes able to inhibit inflammation and bone loss represents an attractive therapeutic strategy in arthritis.

However gene transfer into synoviocytes is limited by the fact that these cells are naturally nonproliferative. As lentiviral vectors are capable of transducing nondividing cells, contrary to the other retroviral vectors, they could represent an interesting solution. Moreover, the recent description of the role in viral DNA nuclear import of a central DNA flap in HIV-1 genome allows the construction of HIV vectors with an increased capacity to introduce genes into nondividing cells. Therefore, we tested such a vector *ex vivo* on human synoviocytes, either nontreated, or growth-arrested by the addition of aphidicolin, or treated with the reverse transcriptase inhibitor AZT. An adenoviral vector was used as positive control, and a murine retroviral vector as negative control. Table 1 shows that the HIV vector, contrary to the control murine retroviral vector, was able to transduce synoviocytes, even in absence of cell division. Lentiviral vectors, that induce no inflammation and are stably integrated, could represent an interesting tool for gene therapy of arthritis. *In vivo* results will be presented.

Table 1

Titer (transducing units/ml)	Synoviocytes	Synoviocytes/ aphidicolin	Synoviocytes/AZT
HIV vector	1.4×10^6	4.5×10^5	$<5 \times 10^3$
Murine retroviral vector	$<5 \times 10^3$	7.5×10^3	$<5 \times 10^3$
Adenoviral vector	1.0×10^7	1.3×10^7	1.0×10^7

P26

Factors influencing adenoviral gene transfer

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Intra-articular injection of an adenoviral vector in nonhuman primates with arthritis results in effecient gene transfer of the synoviocyte like fibroblasts, whereas no gene transfer has been observed to cartilage cells (Goossens *et al*: *Hum Gene Ther* 1999). In humans a possible factor that may influence gene transfer are preexisting antibodies to adenovirus. Now, we have demonstrated that 70% of the synovial fluid samples harvested from 53 different patients inhibited gene transfer of adenovirus.

In 10 samples this was studied in detail by fractionation of the synovial fluid samples. The fraction that inhibited gene transfer most strongly had a molecular mass of 150 kDa and could be purified by protein-A sepharose columns, strongly suggesting that this fraction consisted of antibodies. In order to avoid the influence of naturally occurring autoantibodies we tested if different types of adenoviruses (Ad5, Ad26, Ad34, Ad35 and Ad48) were inhibited as strongly as Ad5. Only 4% of the synovial fluid samples contained antibodies that inhibited Ad35-mediated gene transfer. Next we tested if a vector that contained a fiber from subgroup B (Ad 11, 16 or 35) was as efficient in transducing synoviocytes as the original Ad5 vector. The Ad5-fib35 vector encoding luciferase was about 10 times as efficient than the wild-type fiber when similar particle concentration was used to infect synoviocytes.

In conclusion, the current data suggest that fib35 is efficient in the binding of an adenoviral vector to synoviocyte-like fribroblasts, and that very few RA patients have naturally occurring antibodies to the type 35 adenoviral vector.

P27

Induction of synovial apoptosis by gene transfer and peptide mediated protein transduction

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The destruction of articular cartilage and bone due to pannus formation is one of the more clinically challenging problems associated with rheumatoid arthritis. While synovectomy, the physical removal or killing of synovial tissue, has demonstrated the ability to delay the degradation of the rheumatoid joint the various methods available for this procedure are either highly invasive or are associated with side effects. In order to combat these problems we have been developing a biological synovectomy technique based on the induction of apoptosis. Our approach has focused on the delivery of pro-apoptotic proteins to the diseased synovium either through gene transfer or direct peptide mediated protein delivery. Utilizing a rabbit model of RA we have examined the effects of over expressing a number of proapoptotic proteins (p53, FasL, TRAIL, granzyme B and activated caspase 3) in the cells of the synovial lining. These experiments have demonstrated that the over expression of all of these proteins induce apoptosis in the synovial layer, albeit to differing extents. Intra-articular delivery of adenoviral vectors encoding the proapoptotic genes or proteins fused to peptide transduction domains results in extensive apoptosis in the cells of the synovial lining within 24 h after vector delivery. The induction of apoptosis in the synovium is also associated with a decrease in the inflammation observed in the treated joints. The magnitude of the reduction in leukocytic infiltration correlates directly with the extent of apoptosis induced. These data suggest that the over expression of proapoptotic proteins in synovium may have applications in the treatment of RA.

P28

Targeted adenoviral vectors

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Subgroup C human adenoviruses (Ad2 or Ad5) can transduce a wide array of dividing and quiescent adherent cell types *in vitro* and *in vivo*, essentially because their primary receptor (CAR) is widely expressed at the cellular surface. On the other hand, specific target cell types (eg some tumor cells, smooth muscle cells, fibroblasts) display limiting amounts of the CAR receptor so that large viral doses are needed for efficient infection, a constraint which favors vector dissemination *in vivo*, even after loco-regional injection. Abrogating the native virus-CAR interaction while allowing the virus to use a CAR-independent pathway for entry would thus represent a major milestone in order to better control the vector tropism *in vivo*. Towards this goal, we first engineered a series of capsid-modified adenoviruses by genetically inserting targeting peptides within protruding loops of the fiber and hexon capsid monomers (Vigne E, *et al*: *J Virol* 1999, **73**:5156). The most interesting virus – AE43 – displays within the fiber HI loop a vitronectin-derived high affinity peptide for the urokinase-type plasminogen activator (uPAR or CD87), a cell surface receptor upregulated during cellular activa-

tion, and which controls cellular migration and invasion. Most interestingly, AE43 could increase transduction of cells normally refractory to adenovirus infection more than 100-fold *in vitro*. We could also demonstrate that AE43 retained its ability to enter the cell via a CAR-dependent pathway *in vitro*, and displayed a normal tropism following systemic injection in mice. To cripple the virus-CAR interaction, we then evaluated various strategies, including the introduction of CAR-ablating mutations within the fiber, and the shortening of the fiber shaft. Interestingly, some of these constructs could decrease transduction of CAR-positive cells at least 10-fold *in vitro*, with a similar reduction in liver transduction following systemic injection in naïve mice. We are currently assessing the effect of combining within a single vector the uPAR-binding peptide of AE43 with our best CAR-ablating mutations.

P29

A member of the T-box family of transcription factors mediates cartilage formation in mesenchymal progenitors C3H10T1/2

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The bone morphogenetic protein 2 (BMP2)-dependent onset of osteo-/chondrogenic differentiation in the acknowledged pluripotent murine mesenchymal progenitor cell line C3H10T1/2 is accompanied by the immediate upregulation of fibroblast growth factor receptor 3 (FGFR3) and by a delayed upregulation of FGFR2. This direct FGFR3-mediated expression seems to be essential for the onset of chondrogenesis since the forced expression of activated FGFR3 is sufficient for differentiation into the chondrogenic lineage. The screening for FGFR3-regulated transcription factors exhibiting a chondrogenic capacity in C3H10T1/2 indentified a T-box containing transcription factor. Forced expression of this factor is sufficient for the initiation of chondrogenic differentiation in mesenchymal progenitors C3H10T1/2 *in vitro*. Implantation of these recombinant progenitors at ectopic intramuscular sites of the mouse was followed by massive cartilage formation substantiating the chondrogenic potential of this transcription factor. A potential role for this T-box factor in chondrogenesis is also suggested by its expression in various skeletal elements at late stages of murine embryonic development as demonstrated by *in situ* hybridization. These studies indicate that BMP2 triggers onset of FGFR3-dependent signaling in mesenchymal progenitors C3H10T1/2 to induce a novel type of transcription factor for the initiation of chondrogenic differentiation.

P30

Adenoviral-based overexpression of TIMP-1 reduces tissue damage in the joints of TNF-α transgenic mice

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Chronic inflammation frequently entails the remodelling and/or destruction of the involved tissues. This is illustrated by human diseases and animal models characterized by TNF-α overexpression, such as rheumatoid arthritis and TNF-α transgenic mice, in which severe joint destruction is spontaneously observed in conjunction with chronic synovial inflammation. The detailed molecular mechanisms of TNF-α-mediated tissue damage are, however, unknown. Since matrix metalloproteinases are among the molecules activated by TNF-α we hypothesized that overexpression of their natural inhibitor, tissue inhibitor of metalloproteinases (TIMP)-1, in TNF-α-transgenic mice, could inhibit the development of destructive arthritis. Such adenoviral-based overexpression of TIMP-1 strongly inhibited spreading of inflammation and bone resorption, whereas treatment with the LacZ-control vector had no effect. This inhibition of tissue remodelling and damage by TIMP-1 was also associated with a significant reduction in clinical signs of arthritis and prevention of development of autoimmune phenomena. We conclude that matrix metalloproteinases are major effector molecules of TNF-α-triggered tissue damage and that the highly effective inhibition by TIMP-1 should be considered in the treatment of human disease.

P31

Therapeutic targeting of osteoclasts by osteoprotegerin and bisphosphonates leads to a reduction of TNFα-mediated bone resorption

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Rheumatoid arthritis (RA) is characterized by a cytokine-triggered chronic inflammation and the progressive destruction of bone. TNF-α has been recognized as a crucial inflammatory signal in RA; however, its influence on the destruction of bone is less clear, although it may involve the formation of osteoclasts. In this study, using a TNF-α transgenic mouse model, the effects of osteoclast-targeted therapies, such as osteoprotegerin and pamidronate, were examined on joint inflammation and bone destruction. Mice were divided into five groups receiving either osteoprotegerin, pamidronate, a combination of both agents, infliximab, as a positive control, or phosphate-buffered saline, as a negative control. Treatment was initiated at the onset of arthritis, continued over 6 weeks, and thereafter the clinical, radiological and histological outcomes were assessed. A significant improvement of clinical symptoms, as assessed by the reduction of paw swelling was only found in the infliximab group, whereas all other treatment groups failed to show a significant improvement. However, when assessing structural damage by X-ray analysis, a significant retardation of joint damage was evident in animals treated with osteoprotegerin the combination therapy of osteoprotegerin and pamidronate, and also with infliximab, whereas the reduction of radiologic damage in the pamidronate group was evident, albeit not significant. Quantitative histologic analysis revealed a significant reduction in the size of bone erosions in all treatment groups when compared to the control group. These data suggest that osteoprotegerin alone or its combination with bisphophonates are effective therapeutical tools to prevent TNF-α-mediated destruction of bone.

P32

Gene therapy for cartilage healing

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Articular cartilage has only a limited capacity for repair. Only in the event of damage that extends through to the underlying vasculature and marrow of the subchondral bone will a repair response be initiated; populations of mesenchymal stem cells infiltrate the lesion and synthesize space-filling tissue. This repair tissue is of inferior composition and degenerates with time. Gene transfer strategies, however, may provide methods to stimulate these cells toward the synthesis of more suitable repair tissue that is functionally closer to normal articular cartilage. Through the *in vitro* transfer of genes encoding certain growth factors such as transforming growth factor-β (TGF-β), insulin-like growth factor 1 (IGF-1) and bone morphogenetic protein 2 (BMP-2) we have found it possible to stimulate chondrogenesis of bone marrow stem cells as well as increase the synthesis and deposition of extracellular matrix components by chondrocytes. In contrast to the exploration of complex *ex vivo* methods for engineering cartilaginous tissues, we have been undertaking practical methods for transferring genes encoding chondrogenic growth factors directly to cells infiltrating osteochondral defects in a rabbit model. For this approach, vectors suitable for gene delivery *in vivo* are absorbed into a biologically compatible matrix and implanted directly into freshly generated osteochondral lesions. Using either recombinant adenoviral vectors or plasmid DNAs we have shown that cells entering the defect can infiltrate the implanted matrix, interact with the vector and express the transgene product for up to 3 weeks. This method is currently being evaluated for its effectiveness in repairing osteochondral defects following the delivery of chondrogenic genes such as TGF-β, BMP-2 and IGF-1.

P33

Transfer and intra-articular expression of the IL-1Ra cDNA in human rheumatoid joints

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Permission was received from NIH and the FDA to undertake a phase I clinical study to determine the safety and feasibility of the retroviral, *ex vivo* transfer of a human IL-1Ra cDNA to human rheumatoid joints. Eligible patients were post-menopausal females with end-stage RA requiring replacement of the metacarpophalangeal (MCP) joints of one hand and also requiring surgery on at least one other joint. The latter surgery provided the opportunity to recover autologous synovial tissue from which to establish synovial cell cultures. Half of each patients' cells were transduced with the retrovirus MFG-IRAP, while the other half remained as untransduced controls. After safety testing of both sets of cells, the autologous synoviocytes were injected into the subjects' MCP joints; in a double-blinded manner, two joints received the genetically modified cells, and two joints received control cells. One week later, all four MCP joints were surgically removed during joint replacement surgery and the retrieved joint tissues analyzed for transgene expression. Nine patients were treated in a dose-escalation manner, receiving from 10^6–10^7 cells per MCP joint.

All patients tolerated the procedure well, and no adverse events related to the study were reported. Transcripts originating from the transgene were detected by RT-PCR in all joints that received genetically modified cells, but in only one of the control joints. Analysis of certain samples by *in situ* hybridization and immunohistochemistry confirmed that cells expressing the transgene were located in clumps on the synovial surface. Synovial cells were recovered from the retrieved tissues and placed into cell culture. Concentrations of IL-1Ra were higher in media conditioned by cells recovered from genetically modified joints than in media conditioned by cells from control joints. Although the design of the study precluded assessment of clinical efficacy, several patients reported symptomatic improvement; this was attributed to a placebo effect.

These data confirm that it is possible to transfer genes to human, arthritic joints and to express those genes intra-articularly in a manner that is safe and acceptable to patients. This encourages further development of gene therapies for the treatment of arthritis in humans.

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P34

In vitro and *in vivo* gene delivery using a lentiviral vector

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The delivery of antiarthritic genes to the synovial lining of joints is an effective strategy for the treatment of experimental models of rheumatoid arthritis (RA). Moreover, in two clinical studies it has proved possible to transfer the human interleukin-1 receptor antagonist (hIL-1Ra) cDNA to human rheumatoid joints. These protocols, however, utilized an *ex vivo* approach to gene delivery. While useful for establishing proof of concept, *ex vivo* methods do not lend themselves well to widespread clinical application. For this reason, we are devoting increasing attention to developing clinically acceptable *in vivo* methods of gene delivery to synovium.

Because chronic conditions such as RA will probably require extended periods of intra-articular gene expression, integrating vectors are more attractive. In preclinical experiments, two such vectors, adeno-associated virus and high-titer Moloney-based retrovirus, have shown promise for *in vivo* gene delivery to synovium. Lentiviral vectors also possess favourable properties in this regard, but there are no published data on their suitability for *in vivo* gene delivery to joints. Here we report preliminary data from the use of lentiviruses to deliver genes to articular tissues.

The recent generation of packaging systems able to produce high titers of replication-incompetent HIV-based retroviruses, and the pseudotyping of lentiviral vectors with the vesicular stomatitis virus G-protein (VSV-G) which increase the target cell range and allow concentration by centrifugation have facilitated these studies. In culture we have found that human synoviocytes, and both human and rat chondrocytes were efficiently transduced by high titer ($>10^9$ pfu/ml) of VSV-G pseudotyped HIV-1-based lentiviral vectors containing the β-galactosidase gene (lacZ). Similar patterns of expression were observed using rabbit synovial fibroblast

line, HIG-82, murine 3T3 cells, and primary cultures of rat skin cells. Direct, intra-articular gene delivery was performed by injecting similar lentiviral preparations into the knees of Wistar rats. Histological analyses of the knee joints revealed the expression of lacZ in the synovial membrane for at least one week following injection. No lacZ staining was observed following the injection of empty viruses. Based on the ability to successfully deliver and express the lacZ marker gene in synoviocytes in culture and in the synovial lining *in vivo*, a recombinant vector containing hIL-1Ra has been constructed and is being evaluated.

P35

Engineered cells and cartilage healing

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In chronic joint diseases, inflammation causes an imbalance in cartilage matrix turnover shifting towards degradation. This process may be promoted by destructive invasion of synovial tissue into cartilage as well as a switch in chondrocyte physiology. Furthermore, we could demonstrate a decrease in the expression of morphogenic factors, which are possibly supporting homeostasis and are probably released by the synovium in its function as cartilage nursing tissue. Leading to progressive degradation and loss of the cartilaginous joint surface, chronic joint diseases eventually depend on replacement therapies.

In recent years, methods for biological reconstruction of the articular surfaces with engineered cartilage transplants evolved as an alternative to the established therapy of endoprosthetic arthroplasty. The basic principle of the various strategies is the delivery and integration of functionally active autologous chondrocytes or mesenchymal precursor cells within an appropriate carrier system further supported by differentiation promoting factors into the original anatomic site to restore tissue architecture and function.

Using *in vitro* preformed implants appears to be particularly promising. This cartilage engineering is usually based on application of biocompatible and resorbable embedding substances and/or scaffold materials. Results from gene expression analysis clearly favour three-dimensional instead of monolayer chondrocyte cultivation to enhance cartilage matrix production *in vitro*. Implants of such constructs in the cartilaginous environment of the joint in rabbits or horses were found to produce cartilage typic morphological patterns and matrix synthesis. Heterotopic implantation for example subcutaneously into immunocompromized nude mice may induce unspecific fibroblastoid invasion and implant destruction. Encapsulation experiments prevented this process of infiltration, leading to enhanced matrix production and cartilage formation.

As an alternative and avoiding artificial barriers, tissue maturation and stabilization may be supported by morphogenetic factors, which are representatives of the TGF-β family and key molecules in cartilage and joint formation during development. Clonally expanded bone morphogentic protein (BMP)-7 transgenic primary chondrocytes demonstrated a qualitative switch in collagen expression from type I towards type II when cultured in alginate beads. Other markers of chondrocyte dedifferentiation were down-regulated also. Implantation subcutaneaously into nude mice revealed almost complete exclusion of host fibroblasts from the engineered cartilage accompanied by improved implant maturation.

Thus, the present results demonstrate that current artificial cartilage transplants are already feasible for joint cartilage repair. Nevertheless, treatment of severe joint defects faces specific problems, which are continuously addressed in ongoing studies: the fixation and integration of engineered cartilage in joints; the transplant protection against chronic inflammatory degradation; and the required enormous mechanical stability.

These challenges are particularly addressed by the current developments of composite grafts consisting of bone and cartilage components for reconstruction of the subchondral bone. Furthermore, controlled use of morphogenetic growth factors will unfold great potential to stabilize transplants, promote regeneration and may also allow guided tissue repair starting from mesenchymal precursors or stem cells.

P36

Autologous bone marrow grafting of avascular osteonecrosis before collapse

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Introduction and method: To enhance the rate of bone repair, injection of bone marrow in the necrosis was done during core decompression. The source of bone marrow was the iliac crest. A quantity of 150 ml was obtained by bone marrow aspiration. The whole marrow was concentrated using centrifugation to increase the cell concentration in an aliquot of 25 ml. Bone marrow was injected in the necrosis and in the femoral head by the hole of the core decompression. 42 hips with MRI avascular necrosis without collapse (stage 11) were operated with this technique between 1991 and 1993. The average follow up is 5 years.

Results: Five hips are now failures (collapse) with a stage III at the last revision. The other hips are pain free and stage II. The postoperative MRI demonstrates a modification of the signal in most cases and a disparition of the necrosis in four cases. The number of transplanted cells was calculated in a laboratory study; the number of nucleated cells was counted in the marrow transplanted and the fibroblast colony forming cells (CFU-F) were cloned to appreciate the activity and the number of progenitor in the marrow transplanted ; failures of this technique (five hips with stage 3) occurred in patients with a low number of transplanted cells (average 2000 CFU-F for failures and average 25 000 CFU-F for the other hips).

Conclusion: Many different grafts have been used in avascular necrosis to provide structural support or to enhance bone formation. Since bone marrow contains progenitor cells it may be associated to core decompression. It is a simple and easy adjuvant to core decompression.

P37

Retrovirally-engineered antigen-specific T cells home to the inflamed joints and suppress collagen-induced arthrtis

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Objective: Autoantigen-specific T cells have tissue-specific homing properties, suggesting that these cells may be ideal vehicles for the local delivery of 'immunoregulatory molecules'. We tested this hypothesis by using type II collagen (CII)-specific CD4' T hybridomas, or prim CD4' T cells following gene transfer as vehi-

cles to deliver 'immune regulatory protein' for treatment of collagen-induced arthritis (CIA).
Method: CII-specific T cells were transduced to express IL-12 antagonist IL-12 p4O, using retroviral vectors, and were transferred into CIA mice. To directly examine whether CII-specific T cells home to the site of inflammation, we transduced a GFP-luciferase fusion protein gene into CII-specific T cells and tested the patterns of cell trafficking using whole-body bioluminescence.
Results: Transfer of CII-specific IL12 p4O producing CD4'+T cells after primary immunization significantly inhibited the development of CIA. The beneficial effect of IL-12 p4O-transduced T cells for CIA requires TCR spcificity against CII. Using bioluminescence, we found that CII-reactive T cell hybridomas accumulated and remained in inflamed joints when transferred into CII-immunized arthritic mice.
Conclusion: These results indicated at the local delivery of IL12p4O by T cells inhibited CIA by suppressing an autoimmune response at the site of inflammation. We conclude that modifying antigen-specific T cells by retroviral transduction for local expression of regulatory proteins is a promising therapeutic strategy for the treatment of RA.

P38

Retroviral gene therapy of collagen-induced arthritis by local delivery of IL-4

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Objective: Rheumatoid arthritis (RA) is an autoimmune arthritis for which treatment options remain limited. Pathogenic mechanisms of RA and its animal model collagen-induced arthritis (CIA) involve joint infiltration by pro-inflammatory T-helper 1 (Thl) type $CD4^+$ T-cells. This study investigated the potentiel use of retrovirally transduced collagen type II (CII)-reactive $CD4^+$ T-cells for local delivery of an anti-inflammatory cytokine, interleukin 4 (IL-4), to inflamed joints.
Method: CII-specific $CD4^+$ T-cell hybridomas (made from CII-specific TCR transgenic mouse T cells) were transduced with a retroviral vector encoding IL-4 and the marker yellow fluorescent protein (YFP) in a bicistronic IRES containing construct. Transduced hybridomas were sorted based on YFP expression and injected intravenously into immunized male DBA/lLacJ mice prior to disease onset. For bioluminescence imaging, IL-4 expressing T cell hybridomas were transduced with luciferase expressing retroviral constructs.
Results: Adoptive transfer of transduced hybridomas, that constitutively expressed the transgenes, significantly decreased mean disease severity by reducing the number of inflamed joints. Bioluminescence studies showed that the hybridomas migrated to, accumulated in, and were retained in the inflamed joints. There were no significant changes in the cytokine milieu of the draining lymph nodes nor in the systemic levels of the anti-collagen antibody subtypes IgGl and IgG2a in treated mice.
Conclusion: The beneficial clinical effects observed in our model were most likely based on the local actions of IL-4 in the inflamed joints. The local delivery (and effects) of regulatory cytokines like IL-4 constitute a novel and effective method of treating organ-specific auto-immune diseases and of minimizing the systemic adverse effects of immune-modulating therapy.

P39

Identification and expression of novel genes in osteoarthritis

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Osteoarthritis (OA), the most common form of joint disease, represents a clinical classification of pathological conditions involving progressive degradation of articular cartilage and remodeling of subchondral bone. Genes whose products are involved in chondrogenesis and osteogenesis starting from the common progenitor cells, genes determining the terminal differentiation of chondrocytes and genes whose products trigger breakdown of the cartilaginous matrix are obvious candidates for therapeutic intervention. In this study we used human articular cartilage primary cells to conduct a series of gene expression profiling experiments. The cells were subjected to various treatments, which mimic OA initiation and development: IL-1ß-FAD (FGF-2+Dexamethasone+Ascorbic acid) and mechanical stress. The gene expression profiles corresponding to various applied treatments were studied by microarray hybridization and analyzed by QBI's proprietary bioinformatics tools. The obtained gene expression patterns indicates that the chosen *in vitro* cell system accurately reflects the processes that occur in OA joints *in vivo* since many genes known to be markers of OA were identified by us as displaying the expected type of behavior. After performing the full analysis of hybridization results, a list of 254 genes was obtained. Among them are 210 known genes and 44 novel ones. Out of them 80 genes were further analyzed by *in situ* hybridization on sections obtained from OA patients and control group and by RT-PCR. 15 genes have shown promising results. Among them, genes whose expression was upregulated in chondrocytes located close to the eroded surface of the OA articular cartilage, in osteoprogenitor cells and in endothelial cells. Further studies are being conducted to analyze the role of these genes in OA.

P40

Time-course of gene expression after electroporation in rat patellar chondrocytes

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Objective: The use, via electro-permeabilization (electroporation), of 'chondroprotective' genes may provide a powerful set of tools applicable to the development of plasmid-based gene therapies for OA process. To assess whether permeabilizing drug pulses may overcome the barrier-effect of peri-cellular matrix environment, we investigated the efficiency of plasmid delivery by electroporation and the subsequent duration of transgene expression (GFP and Hsp-70) in rat patellar cartilage.
Methods: The expression vector (pcDNA3.1/CT-GFP) was used to clone the nucleic acid sequence of rat Hsp-70. Empty vector and plasmid carrying the Hsp-70 cDNA were then prepared according to standard procedures. After anesthesia, plasmids were directly injected into both rat knees and transcutaneous electric pulses were then immediately applied (day 0). Expression of GFP and Hsp-70 in rat patellae was studied by immuno-histochemistry from day 2 to 3 months.
Results: On Day 2 after electric pulses, the percentage of GFP positive chondrocytes was about 30% in the superficial cartilage

layer and 60% in the deep layer respectively. No staining was depicted in the intermediate layer. After 3 months, long-term expression of GFP in rat patella was only present in chondrocytes localized in the deep layer (20% of transfected cells). In addition, electric pulses did not alter cartilage structure or metabolism, as assessed by histological evaluation (HES and toluidine blue) and radiolabeled sulfate incorporation. Immuno-staining confirmed the expression of Hsp-70 in a similar pattern than GFP, as observed with the empty vector.

Conclusions: Gene transfer approach, based on the use of electric pulses, is an easy, safe and rapid method that can be used for the direct gene delivery to rat patellar chondrocytes. This method allows targeting of chondrocytes located in the deep layers of cartilage.

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P41

Overexpression and induction of heat shock protein (Hsp) 70 protects *in vitro* and *in vivo* from mono-iodoacetate (MIA)-induced chondrocytes death

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Objective: Cartilage hypocellularity due to cell death contributes to the development of OA. Experimental exposure to MIA results in either chondrocyte necrosis (*in vitro*) or experimental OA with apoptosis when injected *ia* (*in vivo)*. As Hsp-70 is known to protect from cell death, we have evaluated the beneficial properties of its over-expression or induction in rat chondrocyte cells (RCC) after MIA exposure.

Methods: *Cloning* rat Hsp-70 cDNA sequence into the plasmid pcDNA3.1/CT-GFP and transfection led to over expression of Hsp-70 with PEI agent. RT-PCR and Western blotting then monitored expression level of Hsp-70. *In vitro* and *in vivo* exposure of RCC to the proteasome inhibitor MG132 dramatically enhanced the induction of Hsp-70. *Protection* against MIA toxicity was analysed either after transfection of RCC with the vector or after pre-treatment of RCC with MG132. Cytotoxicity of MIA was evaluated by MTT and LDH tests. *In vivo assay*: on day 0, Wistar rats were injected *ia* with MG132, 2 hours before *ia* injection of MIA (0.3 and 0.03 mg). On Day 15, knees and patellae were carefully dissected for histological assessment.

Results: *In vitro* MIA exposure led to cell death, rather by necrosis than by apoptosis (8%). Over-expression of Hsp-70 significantly protected from MIA toxicity in transfected cells after 24 and 48 hours respectively (28 and 64%). Moreover, preventive and curative MG132 (1.5 μM) pre-treatments preserved, at least in part, RCC from MIA chondrotoxicity in a dose-dependant manner related to the magnitude of Hsp-70 induction. *In vivo*, macroscopical evaluation of knees exposed to MIA demonstrated that MG132, when injected *ia* preventively, diminished the severity of OA-like chondral lesions on Day 15.

Conclusions: *In vitro*, the over-expression (gene transfer) or the induction (MG 132) of Hsp-70 protects RCC from MIA cytotoxicity. Induction of Hsp-70 *in vivo* with MG 132 could be promising to modulate OA process and needs to be confirmed in rat experimental OA-models by using gene therapy (viral or non-viral).

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P42

High efficiency intramuscular plasmid electrotransfer: application for sustained plasmatic secretion of cytokines

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Gene delivery to skeletal muscle and to tumors is a promising strategy for the treatment of muscle disorders or cancer, and for the systemic secretion by muscle of therapeutic proteins. We and others have reported efficient plasmid DNA transfer into muscle fibers using square-wave electric pulses of low field strength and of long duration [1–5]. This i.m. plasmid 'electrotransfer' method increases reporter and therapeutic gene expression by several orders of magnitude in various muscles and species, and decreases inter-individual variability. We will present recent results concerning the plasmatic secretion after i.m. gene electrotransfer of a reporter protein, human secreted alkaline phosphatase, which was sustained for more than 12 months. Factor IX was also detected at high concentration in the systemic circulation after i.m. plasmid electrotransfer. Intramuscular electrotransfer of an EPO-encoding plasmid led to a several-months stable hematocrit increase. Moreover, EPO seretion also led to phenotoypic correction in a murine model of beta-thalassemia. Finally, electrotransfer of a murine IL-10-encoding plasmid was shown to induce a significative, but transient increase in circulating IL-10. This had a protective role in atherosclerosis, achieving 60 % reduction in lesion size, and also modulated VEGF expression and neoangiogenesis in a model of hindlimb ischemia.

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