Direct gene delivery strategies for the treatment of rheumatoid arthritis

Steven C. Ghivizzani, Thomas J. Oligino, Joseph C. Glorioso, Paul D. Robbins and Christopher H. Evans

Gene therapy offers a novel and innovative approach to the delivery of therapeutic proteins to the joints of patients with arthritis. Several viral vectors, including adenovirus, adeno-associated virus, retrovirus and herpes simplex virus, are capable of delivering exogenous cDNAs to the synovial lining, enabling effective levels of intra-articular transgene expression following direct injection to the joint. The expression of certain gene products has proven to be sufficient to inhibit the progression of disease in animals with experimental arthritis. Non-viral methods of gene transfer, however, are less satisfactory, and are limited by toxicity and transience of expression. Although the principle of direct gene delivery to the joint has been demonstrated, maintaining persistent intra-articular transgene expression remains a challenge.

***Steven C. Ghivizzani and Christopher H. Evans**
Center for Molecular Orthopaedics
Harvard Medical School
Boston, MA 02115, USA
Thomas J. Oligino, Joseph C. Glorioso and Paul D. Robbins
Department of Molecular Genetics and Biochemistry
University of Pittsburgh School of Medicine
Pittsburgh, PA 15261, USA
*tel: +1 617 732 8607
fax: +1 617 730 2846
e-mail: sghivizzani@rics.bwh.harvard.edu

▼ Rheumatoid arthritis (RA) is a debilitating condition in which the primary symptoms include chronic inflammation of the joints. The pathogenesis of the disease is slow but progressive. The synovial lining of the joint, which is normally a thin layer 2–3 cells deep, becomes dramatically thickened and hypercellular. The constitutive production of inflammatory cytokines in the joint causes the cells in the hypertrophied synovium to become activated. The synovial tissue acquires an aggressive phenotype and invades the articulating tissues, eroding cartilage and subchondral bone. With time, the steady advancement of the disease can lead to a loss of joint function.

To date, RA has proven to be an exceedingly difficult disease to treat. In general, high systemic doses of drugs are necessary to achieve therapeutic levels in the joint, and many agents that are effective in providing symptomatic relief require repeated administration, often with unpleasant side-effects. Advances in molecular biology and biochemistry have enabled the identification of several proteins whose biological properties might be valuable in treating RA[1]. However, these molecules are expensive to manufacture, have limited half-lives *in vivo*, and are difficult to administer effectively.

Gene transfer – an alternative to conventional therapies?

Gene transfer might provide an attractive alternative to conventional drug therapy and drug delivery strategies in the treatment of articular disease. By delivering cDNAs encoding proteins with anti-arthritic or therapeutic properties to certain tissues of the patient, and enabling production of these molecules at elevated levels, it might be possible to achieve sustained therapeutic levels of these agents. If the gene(s) of interest are stably inserted into the tissues, it might be possible to attain long-term relief or even to reverse the pathologies of arthritic disease[2].

Local and systemic gene delivery strategies can be envisaged for the treatment of arthritis. Using a systemic approach, genes would be delivered to the tissues that would permit secreted protein products to readily enter the circulatory system, permitting body-wide distribution and access to all articular tissues[3–9]. With a local approach, exogenous genes would be delivered to cells within specific joints, where the protein products would be synthesized within the joint capsule[10].

Local gene transfer methods

For several reasons, we have directed the majority of our effort toward development of a local gene transfer method. The gene product would be synthesized within afflicted joints,

1359-6446/01/$ – see front matter PII: S1359-6446(01)01679-8

permitting the highest concentration of the protein at the site of disease, thus reducing the risk of exposure to unafflicted tissues and organs. Also, the small fluid volume of the joint space relative to the total human blood volume would require significantly less protein synthesis to achieve therapeutic concentration than a systemic approach. Although originally conceived as a method to treat RA, methods developed for the transfer of genes to specific joints might also have value in the treatment of osteoarthritis (OA), in which typically only a limited number of joints are affected.

The initial studies of gene delivery to the joint employed an *ex vivo* strategy, in which synovial tissue was surgically harvested, and the synovial fibroblasts isolated and cultured. The cultured cells were then infected with a recombinant retrovirus encoding the gene of interest and delivered to the joint by intra-articular injection. The genetically modified cells then colonized the synovial lining and locally expressed the transgene[11]. This procedure proved to be feasible and safe, first in animal models[11–13] and then in a Phase I clinical trial[14].

Following the initial demonstration of gene transfer to the joint, studies of gene therapy for arthritis have, in general, proceeded in two complementary directions. The first is the identification of genes or gene products that exert effective anti-arthritic activity. This usually involves delivering a gene of interest to a particular animal model of arthritis and measuring the effect of its protein expression on the progression of pathology in the experimental system. From these types of experiments, several gene products have been shown to have therapeutic properties when expressed at elevated levels *in vivo*[2]. Second, methods of gene delivery have been evaluated for their relative merit as tools for introducing exogenous DNAs into the joint.

Some studies have investigated the efficiency of intra-articular gene delivery and the persistence of transgene expression in the knee joint of the New Zealand white rabbit rather than small rodents such as rats or mice[11,15–18]. The knee joint of the rabbit is similar in size to many of the human joints that are commonly afflicted with RA and should therefore provide a proportional representation of the effects that can be achieved when treating human disease. The larger size of the rabbit knee also permits accurate and reliable intra-articular injection, and enables the use of serial lavage. The lavage procedure involves the injection of 1 ml saline solution into the joint space, manipulation of the joint to ensure mixing of the saline with the synovial fluid, and then removal of the fluid from the joint with the syringe. Recovered joint washings can be analyzed for the presence and concentration of secreted gene products as well as any pathological responses induced by the gene transfer procedure. Because the lavage procedure can be performed several times without adverse consequence to the animal, the expression of transgenes encoding secreted products can be monitored over time in the same animal. Much of the data discussed in the following paragraphs were obtained using this animal system.

Viral-mediated gene transfer to the joint

Although *ex vivo* delivery provides several potential safety advantages compared with the direct injection of gene transfer vectors to the patient, it is probable that the labor and expense of the procedure would prohibit its use on a large scale. In an effort to streamline the process of intra-articular gene delivery, different means by which cDNAs can be transferred to the synovial lining by the direct intra-articular injection of a gene transfer vehicle or vector have been evaluated, including several viral and non-viral gene transfer methods. Overall, vectors derived from viruses have proven to be the most efficient and permit greater duration of transgene expression. However, each different type of virus that has been adapted for gene transfer has specific advantages and limitations inherent to its physical and biological properties[19].

Adenoviral vectors

Gene transfer vectors derived from replication-deficient adenovirus are the most widely used viral systems for preclinical experimentation owing to their efficient and technically straightforward methods for generating recombinant adenovirus and the comparative ease with which high-titer preparations can be obtained[20–23]. The adenoviral particle is non-enveloped, contains an ~35 kb double-stranded DNA genome and can infect a wide range of cell types from numerous species. Its ability to infect quiescent and dividing cells makes it useful for direct *in vivo* gene delivery. A distinct limitation of the first generation, E1, E3 deleted vectors, which are the most commonly used, is that the majority of native viral-coding sequences are retained and expressed at a low level by virally transduced cells. The production of these viral antigens is thought to contribute, at least in part, to the inflammatory effects often observed *in vivo* following adenoviral-mediated gene delivery[24]. Furthermore, the expression of viral proteins leads to the clearance of transduced cells by the immune system.

Adenoviral vectors will readily infect and efficiently transduce synovial fibroblasts *in vitro*. At a multiplicity of infection between 10 and 100, nearly 100% of the cells in culture can be successfully transduced, often permitting synthesis and secretion of microgram levels of transgene product per milliliter of culture medium per million cells. The direct injection of adenoviral vectors encoding marker

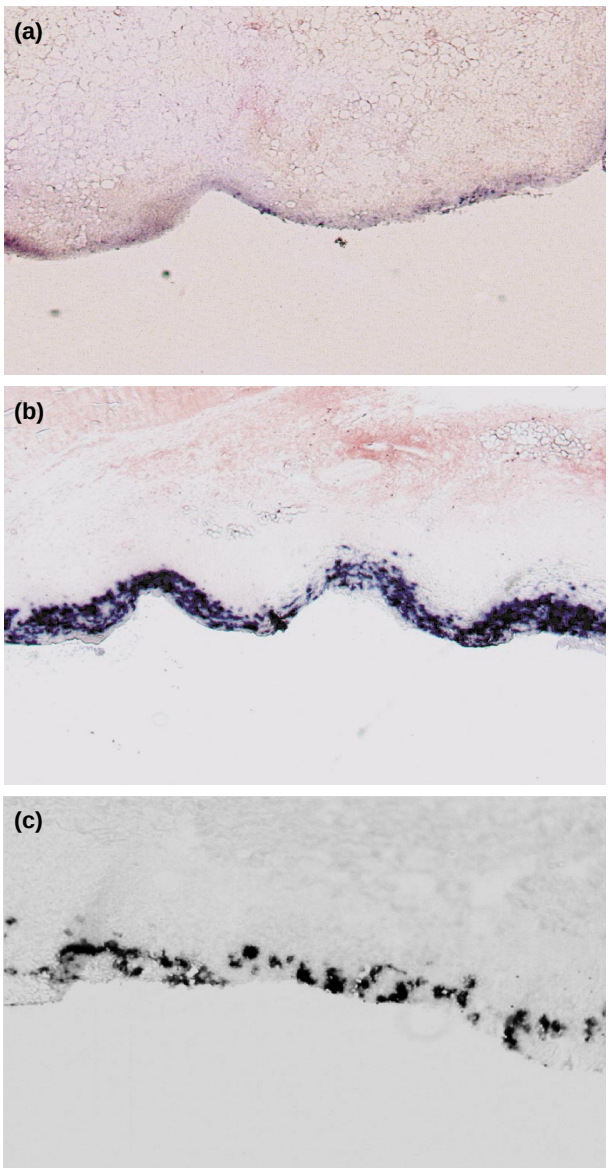

Figure 1. The expression of human alkaline phosphatase (hAP) in the synovial lining of rabbits following adenoviral and non-viral gene delivery. The knees of normal adult rabbits were injected intra-articularly with either 10^{11} particles of a recombinant adenoviral vector [**(b),** magnification × 50; Ad.hAP] or a DNA–liposome formulation [**(c),** magnification × 100; DOTIM–cholesterol]. One week following adenoviral injection, and 24 h after injection of the liposome preparation, the animals were sacrificed and the joint capsules harvested. Frozen sections were prepared from the tissues and stained for hAP activity. Control animals, injected with saline **(a),** magnification × 50, were similarly treated for use as comparative controls.

genes such as lacZ[25,26] or cell-associated alkaline phosphatase into the joints of experimental animals has been found to result in the highly efficient transduction of synovial lining cells, often permitting the genetic modification of cells several layers deep within the lining layer (Fig. 1). The injection of adenoviral vectors encoding various soluble proteins into the rabbit knee has met with the synthesis of tens to hundreds of nanograms of transgene product per milliliter of recovered lavage fluid[16,27].

Although adenoviral vectors have proven to be highly efficient vehicles for gene delivery to the synovium following intra-articular injection, their use has often been associated with the induction of an inflammatory response[5,16]. The intra-articular injection of excessive viral loads or of preparations that have not been sufficiently purified have been observed to induce a nonspecific inflammatory response within 24 h of injection. At a lower dose, the use of first-generation adenovirus will often, within 2–3 weeks, stimulate specific cellular and humoral immunity that results in local inflammation at the site of delivery. In Fig. 2, a typical expression profile observed for adenoviral gene delivery into normal joints is shown. Following the intra-articular injection of increasing amounts of recombinant adenovirus encoding a soluble tumour necrosis factor-alpha (TNFα) receptor, the production and secretion of the transgene is observed in a dose-dependent manner during the first week post-injection. At approximately two weeks, gene expression usually declines and is accompanied by an onset of synovial fluid leukocytosis and mild synovitis. Re-injection of the same adenovirus or an adenoviral vector encoding a different secreted protein does not permit the detection of transgene products.

Using an adenoviral vector, it is possible therefore to achieve high levels of transgene expression intra-articularly that can persist for several days *in vivo*. Even though there are potential inflammatory consequences, this 'window' of intra-articular gene expression has been particularly useful for the screening of genes with anti-arthritic potential. The adenoviral-mediated delivery of several genes encoding secreted gene products, such as the interleukins: IL-10 (Ref. 28), IL-4 (Refs 29,30), vIL-10 (Refs 5,31,32) and IL-1Ra (Ref. 27), among others[2], results in expression levels that are sufficient to block the pathologies of several animal models of arthritis.

Although they are valuable for generating high levels of transient gene expression in the joint, it is highly unlikely that first-generation adenoviral vectors will be used in a clinical setting, particularly in applications that require persistent expression of gene products within the joint. It is possible, however, that later generations of adenoviral vectors, either E1, E4 deleted or 'gutted' vectors that do not contain viral coding regions, might have less of an inflammatory potential and enable longer expression[33]. These types of systems, which have been shown to prolong persistence in certain tissues, have not yet been evaluated in the joint.

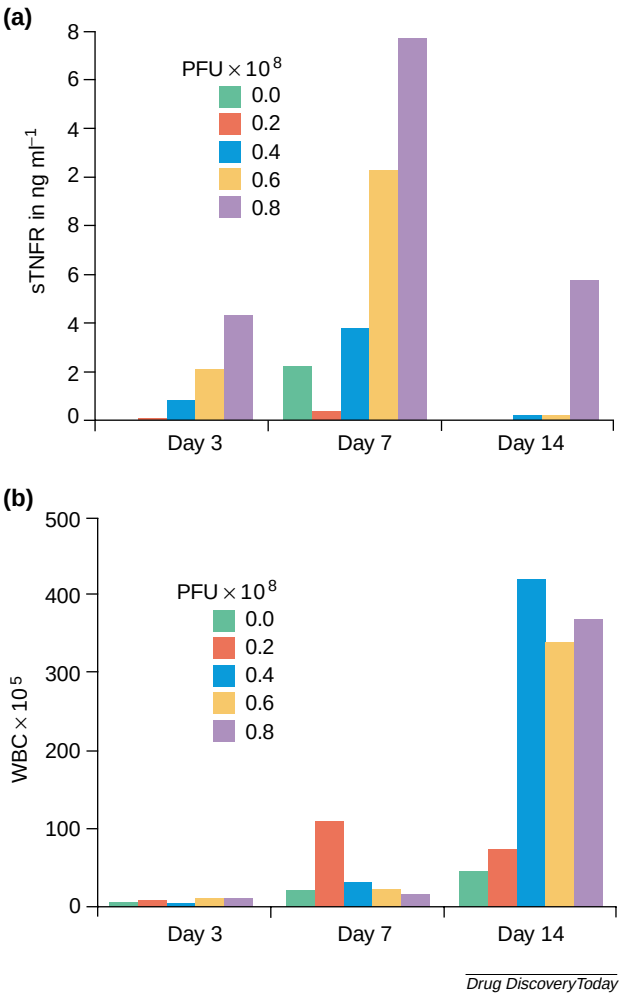

Figure 2. Intra-articular transgene expression and inflammation following adenoviral-mediated delivery of a cDNA encoding a soluble TNF receptor (sTNFR). Increasing doses of a recombinant adenoviral vector encoding a human sTNFR were injected into the knees of normal rabbits. At 3, 7 and 14 days post-injection, the knees of the rabbits were lavaged with saline, and the recovered fluids analyzed for sTNFR levels by ELISA **(a)** and infiltrating leukocytes (WBC) **(b)**.

The delivery of cytotoxic or apoptotic genes to invasive synovium might be an alternative approach for which adenoviral vectors are well suited. Because the phenotype of rheumatoid synovium resembles in many ways that of an aggressive tumor, it might be possible to adapt certain gene therapy strategies that were originally devised for treating cancer to RA. By delivering genes to the joint that encode proteins capable of killing the cells within the synovium, it might be possible to delay or slow the destructive progression of the disease. cDNAs encoding Fas ligand[34,35] (FasL) and herpes simplex virus thymidine kinase[36,37] (HSVtk) have been successfully transferred to cells in the synovial lining and have been shown to mediate significant cell death. Adenoviral vectors are unlikely to be useful in settings where long-term gene expression is needed; however, approaches such as these, which rely on a high efficiency of gene delivery but require only a transient burst of expression, might prove to be viable clinical applications of adenoviral-mediated gene transfer to the joint.

Retroviral vectors

Retroviral vectors derived from the Moloney murine leukemia virus (MoMLV) have been used extensively in the laboratory and in the majority of gene therapy clinical trials. Several features of the MoMLV-based vectors have contributed to their popularity as a means of gene delivery[19]. They have an ample, 8–10 kb, capacity for exogenous DNA, and do not retain any of the native viral coding sequences. Because this virus is capable of integrating its genome into that of the host cell, the gene of interest will persist for the life of the transduced cell and will be present in all daughter cells. Thus, if the proper progenitor or cell population were successfully modified with this vector system, it might be possible to achieve stable transgene expression *in vivo*. Further, when attempting to treat chronic persistent afflictions such as RA and OA, enduring transgene expression will perhaps be necessary. Because MoMLV-based retroviral vectors require mitosis for the successful transduction of target cells, the use of these vectors has been, for the most part, limited to *ex vivo* gene delivery. A possible drawback associated with the use of these vectors is the potential for insertional mutagenesis, which might occur following the random integration of the provirus into the host genome. To date, however, no adverse clinical response has been reported with the use of this vector system.

In certain cases, where local cell division can be induced *in vivo* (e.g. in the liver after partial hepatectomy[38]), it has been possible to achieve significant levels of transduction *in vivo* following local infusion of recombinant retroviral particles. We hypothesized that under conditions of acute intra-articular inflammation, in which the synovial lining becomes hypercellular from proliferating synoviocytes, the local cell division in this environment might support appreciable retroviral-mediated gene delivery. Indeed, following the onset of IL-1-induced arthritis in the rabbit knee, injection of high-titer (>10^8 infectious particles per milliliter) recombinant retroviral preparations encoding human growth hormone as a secretable marker was found to generate levels of secreted transgene product comparable to that achieved with *ex vivo* gene delivery[15]. Related experiments in rats with adjuvant-induced arthritis also found significant transfer of a lacZ marker gene to inflamed synovium using a concentrated retroviral vector[39]. Expression

was noted to peak at 3–7 days post-injection but was detected for up to seven weeks in this animal system. Transgene expression following intra-articular injection was limited to the synovium and was not detected in organs or tissues outside of the injected joints.

To date, high-titer preparations of MoMLV-based vectors have been difficult to generate routinely, and this has limited studies involving direct retroviral-mediated gene delivery. Advances in technology might help to expand the practical use of retrovirus beyond *ex vivo* applications. For example, the ability to produce pseudotype retrovirus, where the vesicular stomatitis virus G-protein is incorporated into the MoMLV viral envelope[40], has provided increased infectivity of the vector and the ability to concentrate retroviral particles by ultracentrifugation. In addition, the development of lentiviral-based vectors,[41] which are able to transduce non-mitotic cells, might further enable the advantages of retroviral-mediated gene transfer to be adapted to direct gene delivery to the joint.

Herpes simplex virus

The herpes simplex virus (HSV) offers several potential benefits that could be particularly useful for treating arthritic conditions[42]. Within the large 152 kb HSV genome, 43 out of the 81 known coding sequences are non-essential for replication *in vitro*. This enables ample room for the insertion of multiple genes and complex regulatory regions. Because the cytokine network that participates in the inflammatory response is complex, the successful treatment of RA might involve the delivery of several types of proteins and require controlled coordinate expression. HSV also has the ability to develop latency in certain cell types, in which the viral genome persists for the life of the host cell without integrating into the host genome and without altering host cell metabolism. A neuronal-specific promoter system that is uniquely capable of remaining active during latency, when all other viral gene promoters are repressed, has been shown to express foreign genes during latency[43]. Replication-defective viruses can be constructed that establish latency even though they are unable to replicate *in vivo*, and this pseudo-latent state can occur in a variety of cell types in addition to neurons.

First-generation, replication-defective HSV-based vectors were derived by the inactivation of the immediate-early, infected cell protein 4 (ICP4; Ref. 44). The delivery of exogenous cDNAs to rabbit synovial fibroblast cultures via this viral system proved to be highly efficient; however, the continued synthesis of other HSV immediate-early proteins was highly cytotoxic. Later-generation vectors, deficient for ICP4, 22 and 27 and UL41 (Ref. 45) were found to be significantly improved and permitted persistent expression *in vitro* without evidence of cell death[17]. The delivery of this vector encoding human IL-1Ra to the joints of rabbits with experimental arthritis was found to generate nanogram levels of secreted protein, sufficient to ameliorate certain inflammatory effects of the arthritis model. This elevated expression was found to persist for ~7 days, after which gene expression was rapidly lost and accompanied by leukocytic infiltration of the synovial fluid. Although most of the immediate-early genes are inactivated, similar to adenovirus, HSV vectors also permit low level expression of certain viral proteins and, thereby, have an inflammatory capacity. The re-injection of the HSV vector after the loss of expression has only been met with detectable transgene expression in a limited number of animals. Transgene expression has not been observed in any animal following a third intra-articular injection[17]. Consequently, although HSV vectors have features that might be advantageous for RA gene therapy, further development will be necessary before its administration to human joints.

Adeno-associated virus

The adeno-associated virus (AAV) also has certain characteristics that favor its use as a gene delivery vector to the tissues of the joint. Wild-type AAV is non-pathogenic and is not associated with any known disease. For gene delivery, recombinant AAV is engineered so that it encodes no viral proteins, reducing the immunogenicity of the transduced cell *in vivo* and its capacity to stimulate an inflammatory response. Because the virus infects a wide variety of dividing and non-dividing cells, it can achieve significant levels of cellular transduction following delivery *in viv*o, and, in some tissues, recombinant AAV has been found to integrate into the genome of the target cell enabling persistent gene expression[46–48]. Recent advances in methods for generating large-scale, high-titer, adenovirus-free preparations[49–51] have brought wider interest to the use of this vector system, including its potential for use in treating the arthritides (arthritic conditions, such as RA and OA). Indeed, an initial study using an AAV vector encoding β-galactosidase as a marker gene has indicated that gene expression following intra-articular injection is significantly higher in inflamed joints than in normal joints[52]. Further, it was found that gene expression, once lost, could be rescued by the induction of a second inflammatory episode.

Patterns of AAV-mediated gene expression following intra-articular injection of AAV encoding IL-1Ra into normal and inflamed joints of rabbits have recently been characterized (Oligino and colleagues, unpublished). No significant difference was found in the levels or duration of expression of the IL-1Ra transgene between the two groups. Typically, following injection of ~5×10^{11} particles,

approximately one nanogram of IL-1Ra was detected per milliliter of recovered lavage fluid for the first week post-injection. During this period, a significant reduction in the leukocytic infiltration was observed in joints inflamed by constitutive IL-1 production. After the first week, expression was found to gradually diminish, and in most animals was absent by 21 days. In several animals, expression persisted for a longer time period and was diminished at 4–5 weeks. It was found that following the loss of IL-1Ra transgene expression, neither re-injection of the AAV.IL-1Ra vector nor the induction of an inflammatory response could generate detectable levels of IL-1Ra in recovered lavage fluids. In normal animals injected with the AAV.IL-1Ra virus, no evidence of an inflammatory response was observed at any of the time points analyzed.

Several studies of AAV-mediated gene transfer to non-articular tissues have been reported to exhibit persistent, if not stable, transgene expression *in vivo*[53–55]. In some cases, expression has been found to remain for greater than a year[56–58]. In joint tissues, however, AAV-mediated gene expression is of limited duration. There are several possible explanations that might account for this difference, including synovial cell turnover, failure of integration and loss of viral DNA, or immune response to expression of a human protein in the rabbit. It has been reported that the expression of an exogenous transgene product in an adenoviral infection can stimulate a potent immune response to the expressed protein; AAV, however, has not been shown to augment this process[59]. In the rabbit knee, although gene expression gradually diminished, unlike adenovirus, no accompanying leukocytosis was observed, suggesting that a strong immune reaction was not initiated to the transduced cells.

Overall, there is cautious optimism toward the potential use of AAV-based vectors in treating articular disease. As shown by the ability of the IL-1Ra expression to alleviate leukocytosis in the inflamed joints of rabbits, the efficiency of AAV-mediated gene delivery and expression in the joint is sufficient to induce a biological response in a joint of human proportion. The observation that cells within both normal and inflamed joints are similarly capable of being transduced by an AAV-based vector and of expressing a transgene indicates that this type of vector might have application in a broad spectrum of articular ailments. These include inflammatory conditions, such as RA, in addition to those not directly associated with inflammation (e.g. OA) and the repair of joint tissues such as meniscus and ligament[60].

Non-viral gene delivery to synovium

Although viral gene transfer approaches have been shown for the most part to be safe (>3000 patients have been successfully treated until 1999), numerous safety concerns make their use unappealing. With the use of non-viral, plasmid-based systems, no infectious agents are administered to the patient or to the patient's cells, eliminating a large portion of the potential risks associated with gene transfer[61]. In an effort to devise a safer method for delivering exogenous genes to joints, we undertook the large-scale screening of a wide variety of DNA formulations.

Evaluating DNA formulations for gene delivery

Our strategy was adapted from our previous experiments with viral vectors in the rabbit knee system. Owing to the availability of a reliable ELISA, and our experience in preclinical animal work and in gene transfer to human joints, human IL-1Ra was selected for use as a secretable marker for transgene expression. In earlier studies involving the direct injection of various viral vectors, levels of secreted transgene products in lavage fluids recovered from rabbit knees were observed to range up to several hundred nanograms of IL-1Ra per millilitre of recovered fluid, depending on the type and amount of virus injected. Investigations of intra-articular transfer of various therapeutic genes into either antigen- or IL-1-induced models of arthritis in the rabbit knee indicated that, in general, protein levels of at least 1 ng ml^{-1} of lavage fluid were necessary to achieve an observable biological response. Thus, in evaluating the potential of various DNA formulations, this level of expression was established as a working benchmark[11,12,17]. In preliminary work with naked DNA and a common DNA-liposome formulation (DOTIM-cholesterol), it was found that with either preparation, injection of 0.5 ml of a 1 mg ml^{-1} DNA preparation was necessary to achieve the minimal level of gene expression in the rabbit knee joint. Parallel injections of plasmid DNAs encoding cell-associated human alkaline phosphatase as a histological marker demonstrated that a large number of cells in the synovial lining could be genetically modified by this procedure (Fig. 1). Together, these observations suggested that it was indeed possible to achieve significant gene transfer to the synovial lining by the non-viral approach[18].

Before initiating large-scale screening of different DNA formulations in animals, exhaustive efforts were made to identify *in vitro* parameters that are useful in selecting candidate formulations with increased likelihood of efficient gene delivery *in vivo*. Unfortunately, however, there proved to be no correlation between transfection efficiency *in vitro* and *in vivo* in the rabbit knee. The *in vivo* strategy was straightforward: a low number of HIG-82-IL-1+ cells[62] were injected into both knee joints of rabbits to induce a mild inflammatory response (in more recent work, it was found that there was no significant difference in gene transfer

into normal or the mildly inflamed knees so this portion of the protocol was discontinued). Two to three days later, candidate DNA formulations were injected into both knee joints of several rabbits; at 1, 3 and 7 days post-injection, the rabbit knees were lavaged. From the recovered fluids, infiltrating leukocytes were determined and the level of IL-1Ra was measured using ELISA[18].

During the screening procedure, >75 different DNA formulations were evaluated in the rabbit knees. These formulations included naked DNA, cationic and anionic liposomes, as well as various polymers and peptides. At the completion of the screening, only a few formulations were found to be capable of achieving the nanogram level of IL-1Ra expression. This was largely predicted, given the reduced efficiency reported for non-viral gene transfer. The transience of IL-1Ra expression and the level of inflammation induced by intra-articular injection of the non-viral preparations were less anticipated (Fig. 3). As shown in Fig. 3a, significant levels of IL-Ra synthesis were detected 24 h post-injection, but by 48 h, IL-Ra levels in lavage fluids had fallen to background levels (<100 pg ml^{-1}). Surprisingly, levels of infiltrating white blood cells in the synovial fluid of the knees receiving DNA, either alone or in any formulation, greatly surpassed that obtained with the IL-1 model, in some cases by as much as 10-fold. In most of the experiments, the inflammation persisted for at least 1 week, far exceeding the duration of expression of the therapeutic gene. The repeat administration of formulations capable of sufficient gene expression was associated with increasingly severe levels of inflammation and the development of large osteophytes[18].

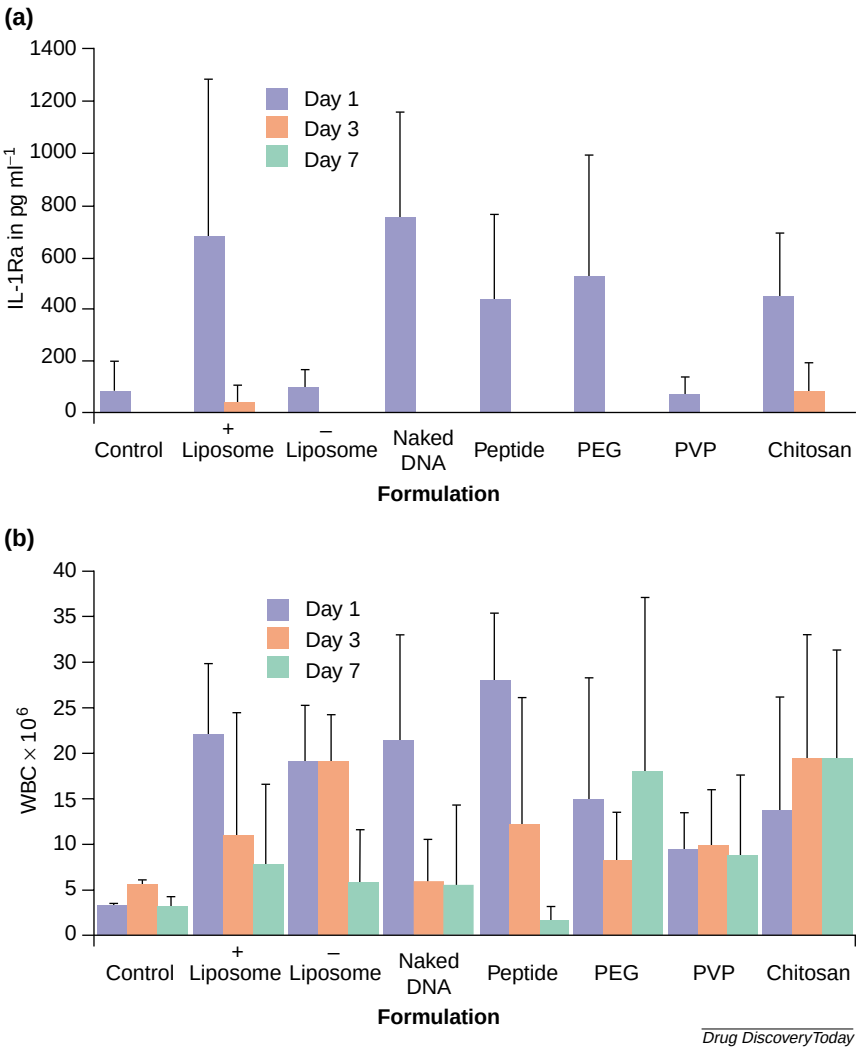

Figure 3. Intra-articular transgene expression and inflammation following non-viral-mediated delivery of the human interleukin-1 receptor antagonist (IL-1Ra) cDNA to the joints of rabbits. Normal rabbits were injected intra-articularly with plasmid DNA formulated with lipid [liposome, plus (+) or minus (–) the IL-1Ra coding region], an oligomeric peptide, various polymers (polyethylene glycol, PEG; polyvinylpyrrolidone, PVP, or chitosan) or unformulated (naked). In all subjects, 0.5 mg DNA was injected. At 1, 3 and 7 days post-injection, the animals were lavaged with saline and the recovered fluids analyzed for human IL-1Ra content by ELISA **(a)** and infiltrating leukocytes (WBC) **(b)**. Control animals were injected with similar volumes of saline solution. For each formulation, both knees of three rabbits were injected and the mean value (n = 6) is shown for each bar. Error bars represent one standard deviation from the mean.

These results are in contrast to experiments reported by Fernandes and coworkers[63] in which milligram quantities of a lipid–DNA complex encoding canine IL-1Ra were injected into the joints of rabbits that had undergone a surgically induced model of OA. In this study, significant improvements were observed several weeks following injection, which correlated with administration of the IL-1Ra plasmid. Inflammatory responses following *in vivo* delivery of DNA and lipid–DNA complexes have been reported by several laboratories following intravenous[64], intraperitoneal[65] and intratracheal[66] injections. The exact mechanism is currently unknown but might be related to the presence of unmethlyated CpG motifs in plasmid DNAs propagated in bacteria. A recent study by Norman

and colleagues[65] showed that intraperitoneal administration of liposome–DNA complexes stimulates production of IL-1β and TNFα and causes an increase in serum acute-phase proteins. Furthermore, this process was shown to significantly enhance pre-existing inflammation in a murine pancreatitis model.

The results of our experiments with non-viral delivery provide limited optimism that non-viral gene delivery will be useful for treating human articular conditions. A major shortfall of non-viral gene delivery approaches is typically that transfection efficiency *in vivo* is relatively low. We have found that it is at least possible to achieve functional levels of transgene expression using this technology, but the inflammation and transience of gene expression remain as immediate barriers.

Directions for the future

It has been shown that exogenous cDNAs can be efficiently delivered to cells within the synovial lining by direct intra-articular injection of certain viral and non-viral vectors. Following delivery, the expression of specific transgene products is sufficient to elicit beneficial responses in several animal models of arthritis. Further, via *ex vivo* (whereby tissue from a patient is removed, manipulated, and then returned to the patient) gene delivery, successful, safe, gene transfer has been demonstrated in the joints of humans. Thus, the principle of gene therapy for arthritis has been proven.

Considerable work remains, however, if gene therapy is to move beyond the experimental and into the practical arena. Although much progress has been made, particularly in the area of identification of genes with anti-arthritic potential, similar to the field of gene therapy in general, the development of a satisfactory method of gene transfer remains the greatest impediment. Although efficiency of delivery has proven to be sufficient, persistent expression remains a challenge. In virtually all studies performed to date, gene expression within the synovium has been found to be transient, persisting at a high level for no longer than 2–3 weeks.

Perhaps the best place to begin to address the issue of persistence of transgene expression is in the basic biology of the synovium. Unfortunately, few data are available concerning such fundamental issues as the rate or mechanism of cellular turnover in this tissue and the source of synoviocyte progenitors. Understanding more clearly the nature of the synovium will help to determine the types of genes and gene delivery strategies with the greatest potential for successful application. Furthermore, to clearly evaluate different methods of gene delivery and available vectors, it is crucial to develop homologous animal systems within which to conduct experiments.

To date, the majority of studies related to gene therapy for arthritis have involved an interplay of non-homologous systems, whereby a gene from one species (or phylogenetic kingdom) has been delivered to that of another. To resolve issues related to the transient persistence of expression, it will be necessary to unambiguously identify the source(s) of the problems. Because proteinaceous transgene products will be presented to the immune system in the context of class I major histocompatibility molecules, it is essential that cells genetically modified to express these molecules are not interpreted as non-self and subsequently targeted for elimination. Thus, to firmly define the specific benefits and limitations of various vector systems and target tissues it will be necessary to evaluate them using marker genes that are completely native to the animal model. Further, it is imperative that the markers used are reliable and can be unambiguously and reproducibly measured against the background of endogenously produced protein.

References

1 Breedveld, F.C. (1999) Future trends in the treatment of rheumatoid arthritis: cytokine targets. *Rheumatology* 38 (Suppl. 2), 11–13

2 Evans, C.H. *et al.* (1999) Gene therapy for rheumatic diseases. *Arthritis Rheum.* 42, 1–16

3 Le, C.H. *et al.* (1997) Suppression of collagen-induced arthritis through adenovirus-mediated transfer of a modified tumor necrosis factor alpha receptor gene. *Arthritis Rheum.* 40, 1662–1669

4 Bessis, N. *et al.* (1996) Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13. *Eur. J. Immunol.* 26, 2399–2403

5 Ma, Y. *et al.* (1998) Inhibition of collagen-induced arthritis in mice by viral IL-10 gene transfer. *J. Immunol.* 161, 1516–1524

6 Mageed, R.A. *et al.* (1998) Prevention of collagen-induced arthritis by gene delivery of soluble p75 tumour necrosis factor receptor. *Gene Ther.* 5, 1584–1592

7 Apparailly, F. *et al.* (1998) Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. *J. Immunol.* 160, 5213–5220

8 Jorgensen, C. *et al.* (1999) Systemic viral interleukin-10 gene delivery prevents cartilage invasion by human rheumatoid synovial tissue engrafted in SCID mice. *Arthritis Rheum.* 42, 678–685

9 Kim, K.N. *et al.* (2000) Viral IL-10 and soluble TNF receptor act synergistically to inhibit collagen-induced arthritis following adenovirus-mediated gene transfer. *J. Immunol.* 164, 1576–1581

10 Evans, C.H. and Robbins, P.D. (1999) Gene therapy of arthritis. *Intern. Med.* 38, 233–239

11 Bandara, G. *et al.* (1993) Intraarticular expression of biologically active interleukin 1-receptor-antagonist protein by *ex vivo* gene transfer. *Proc. Natl. Acad. Sci. U. S. A.* 90, 10764–10768

12 Otani, K. *et al.* (1996) Suppression of antigen-induced arthritis in rabbits by *ex vivo* gene therapy. *J. Immunol.* 156, 3558–3562

13 Makarov, S.S. *et al.* (1996) Suppression of experimental arthritis by gene transfer of interleukin 1 receptor antagonist cDNA. *Proc. Natl. Acad. Sci. U. S. A.* 93, 402–406

14 Evans, C.H. *et al.* (1996) Clinical trial to assess the safety, feasibility, and efficacy of transferring a potentially anti-arthritic cytokine gene to human joints with rheumatoid arthritis. *Hum. Gene Ther.* 7, 1261–1280

15 Ghivizzani, S.C. *et al.* (1997) Direct retrovirus-mediated gene transfer to the synovium of the rabbit knee: implications for arthritis gene therapy. *Gene Ther.* 4, 977–982

16 Ghivizzani, S.C. *et al.* (1998) Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to

rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc. Natl. Acad. Sci. U. S. A.* 95, 4613–4618
17 Oligino, T. *et al.* (1999) Intra-articular delivery of a herpes simplex virus IL-1Ra gene vector reduces inflammation in a rabbit model of arthritis. *Gene Ther.* 6, 1713–1720
18 Oligino, T.C. *et al.* (2000) Vector systems for gene transfer to joints. *Clin. Orthop.* 379 (Suppl.), S17–S30
19 Robbins, P.D. and Ghivizzani, S.C. (1998) Viral vectors for gene therapy. *Pharmacol. Ther.* 80, 35–47
20 Hitt, M. *et al.* (1994) Construction and Propagation of Human Adenovirus Vectors. In *Cell Biology: A Laboratory Handbook* (Celis, J., ed.), pp. 479–490, Academic Press
21 Yeh, P. and Perricaudet, M. (1997) Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J.* 11, 615–623
22 Hardy, S. *et al.* (1997) Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* 71, 1842–1849
23 He, T.C. *et al.* (1998) A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. U. S. A.* 95, 2509–2514
24 Ginsberg, H.S. (1996) The ups and downs of adenovirus vectors. *Bull. New York Acad. Med.* 73, 53–58
25 Roessler, B.J. *et al.* (1993) Adenoviral-mediated gene transfer to rabbit synovium *in vivo*. *J. Clin. Invest.* 92, 1085–1092
26 Nita, I. *et al.* (1996) Direct gene delivery to synovium. An evaluation of potential vectors *in vitro* and *in vivo*. *Arthritis Rheum.* 39, 820–828
27 Roessler, B.J. *et al.* (1995) Inhibition of interleukin-1-induced effects in synoviocytes transduced with the human IL-1 receptor antagonist cDNA using an adenoviral vector. *Hum. Gene Ther.* 6, 307–316
28 Lubberts, E. *et al.* (2000) Intra-articular IL-10 gene transfer regulates the expression of collagen-induced arthritis (CIA) in the knee and ipsilateral paw. *Clin. Exp. Immunol.* 120, 375–383
29 Lubberts, E. *et al.* (1999) Adenoviral vector-mediated overexpression of IL-4 in the knee joint of mice with collagen-induced arthritis prevents cartilage destruction. *J. Immunol.* 163, 4546–4556
30 Lubberts, E. *et al.* (2000) IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. *J. Clin. Invest.* 105, 1697–710
31 Whalen, J.D. *et al.* (1999) Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. *J. Immunol.* 162, 3625–3632
32 Lechman, E.R. *et al.* (1999) Direct adenoviral gene transfer of viral IL-10 to rabbit knees with experimental arthritis ameliorates disease in both injected and contralateral control knees. *J. Immunol.* 163, 2202–2208
33 Robbins, P.D. *et al.* (1998) Viral vector for gene therapy. *Trends Biotechnol.* 16, 35–40
34 Zhang, H. *et al.* (1997) Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer. *J. Clin. Invest.* 100, 1951–1957
35 Yao, Q. *et al.* (2000) Adenoviral mediated delivery of FAS ligand to arthritic joints causes extensive apoptosis in the synovial lining. *J. Gene Med.* 2, 210–219
36 Sant, S.M. *et al.* (1998) Molecular lysis of synovial lining cells by *in vivo* herpes simplex virus-thymidine kinase gene transfer. *Hum. Gene Ther.* 9, 2735–2743
37 Goossens, P.H. *et al.* (1999) Feasibility of adenovirus-mediated nonsurgical synovectomy in collagen-induced arthritis-affected rhesus monkeys. *Hum. Gene Ther.* 10, 1139–1149
38 Kay, M.A. *et al.* (1992) Hepatic gene therapy: persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery *in vivo*. *Hum. Gene Ther.* 3, 641–647
39 Nguyen, K.H. *et al.* (1998) Direct synovial gene transfer with retroviral vectors in rat adjuvant arthritis. *J. Rheumatol.* 25, 1118–1125
40 Emi, N. *et al.* (1991) Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *J. Virol.* 65, 1202–1207
41 Trono, D. (2000) Lentiviral vectors: turning a deadly foe into a therapeutic agent. *Gene Ther.* 7, 20–23
42 Evans, C. *et al.* (1997) Progress in development of herpes simplex virus gene vectors for treatment of rheumatoid arthritis. *Adv. Drug. Deliv. Rev.* 27, 41–57
43 Goins, W.F. *et al.* (1997) Herpes simplex virus vectors for gene transfer to the nervous system. *J. Neurovirol.* 3 (Suppl. 1), S80–88
44 Glorioso, J.C. *et al.* (1994) Herpes simplex virus vectors and gene transfer to brain. *Dev. Biol. Stand.* 82, 79–87
45 Krisky, D.M. *et al.* (1998) Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther.* 5, 1593–1603
46 Yang, C.C. *et al.* (1997) Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration *in vivo* and *in vitro*. *J. Virol.* 71, 9231–9247
47 Malik, P. *et al.* (1997) Recombinant adeno-associated virus mediates a high level of gene transfer but less efficient integration in the K562 human hematopoietic cell line. *J. Virol.* 71, 1776–1783
48 Rutledge, E.A. and Russell, D.W. (1997) Adeno-associated virus vector integration junctions. *J. Virol.* 71, 8429–8436
49 Xiao, X. *et al.* (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.* 72, 2224–2232
50 Clark, K.R. *et al.* (1999) Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum. Gene Ther.* 10, 1031–1039
51 Gao, G.P. *et al.* (1998) High-titer adeno-associated viral vectors from a Rep/Cap cell line and hybrid shuttle virus. *Hum. Gene Ther.* 9, 2353–2362
52 Pan, R.Y. *et al.* (1999) Disease-inducible transgene expression from a recombinant adeno- associated virus vector in a rat arthritis model. *J. Virol.* 73, 3410–3417
53 Snyder, R.O. *et al.* (1997) Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.* 16, 270–276
54 Flotte, T.R. *et al.* (1993) Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector. *Proc. Natl. Acad. Sci. U. S. A.* 90, 10613–10617
55 Herzog, R.W. *et al.* (1997) Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5804–5809
56 Xiao, X. *et al.* (1996) Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J. Virol.* 70, 8098–8108
57 Guy, J. *et al.* (1999) Reporter expression persists 1 year after adeno-associated virus-mediated gene transfer to the optic nerve. *Arch. Ophthalmol.* 117, 929–937
58 Chao, H. *et al.* (1999) Persistent expression of canine factor IX in hemophilia B canines. *Gene Ther.* 6, 1695–1704
59 Jooss, K. *et al.* (1998) Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J. Virol.* 72, 4212–4223
60 Evans, C.H. and Robbins, P.D. (1999) Genetically augmented tissue engineering of the musculoskeletal system. *Clin. Orthop.* 367, S410–418
61 Li, S. and Huang, L. (2000) Nonviral gene therapy: promises and challenges. *Gene Ther.* 7, 31–34
62 Ghivizzani, S.C. *et al.* (1997) Constitutive intra-articular expression of human IL-1 beta following gene transfer to rabbit synovium produces all major pathologies of human rheumatoid arthritis. *J. Immunol.* 159, 3604–3612
63 Fernandes, J. *et al.* (1999) *In vivo* transfer of interleukin-1 receptor antagonist gene in osteoarthritic rabbit knee joints: prevention of osteoarthritis progression. *Am. J. Pathol.* 154, 1159–1169
64 Li, S. *et al.* (1999) Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am. J. Physiol.* 276, L796–804
65 Norman, J. *et al.* (2000) Liposome-mediated, nonviral gene transfer induces a systemic inflammatory response which can exacerbate pre-existing inflammation. *Gene Ther.* 7, 1425–1430
66 Yew, N.S. *et al.* (2000) Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol. Ther.* 1, 255–262