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Perspectives on the Use of Gene Therapy for Chronic Joint Diseases

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Abstract

Advances in molecular and cellular biology have identified a wide variety of proteins including targeted cytokine inhibitors, immunomodulatory proteins, cytotoxic mediators, angiogenesis inhibitors, and intracellular signalling molecules that could be of great benefit in the treatment of chronic joint diseases, such as osteo- and rheumatoid arthritis. Unfortunately, protein-based drugs are difficult to administer effectively. They have a high rate of turnover, requiring frequent readministration, and exposure in non-diseased tissue can lead to serious side effects. Gene transfer technologies offer methods to enhance the efficacy of protein-based therapies, enabling the body to produce these molecules locally at elevated levels for extended periods. The proof of concept of gene therapies for arthritis has been exhaustively demonstrated in multiple laboratories and in numerous animal models. This review attempts to condense these studies and to discuss the relative benefits and limitations of the methods proposed and to discuss the challenges toward translating these technologies into clinical realities.

Keywords

Arthritis; gene therapy; adenovirus; adeno-associated virus; lentivirus; osteoarthritis; interleukin-1; tumor necrosis factor

INTRODUCTION

In recent years, numerous biological agents have been identified with the potential to modulate the pathologic processes associated with chronic joint conditions. Both protein and nucleic acid based agents have been used to block inflammation, cartilage and bone degeneration, and fibrosis; others have been found with the capacity to accelerate tissue healing and repair.

An indication of the clinical potential to be gained from the use of protein-based therapies is provided by various tumor necrosis factor (TNF) inhibitors in the treatment of rheumatoid arthritis (RA). Molecules such as etanercept [1], a modified soluble TNF cell-surface receptor fused to an immunoglobulin domain, and adalimumab and infliximab [2, 3], engineered anti-TNF antibodies, are presently among the most effective medicines for RA. Although these recombinant proteins represent a significant medical advance, they have considerable drawbacks. Protein-based therapies are very expensive [4], and because proteins typically have limited half-lives *in vivo*, they require frequent administration, presumably for the lifetime of the patient. While these agents are an improvement over pre-existing conventional pharmacologics, they still only provide partial relief from disease symptoms, and many patients are unresponsive to treatment. Furthermore, the systemic route of administration is associated with side effects in some patients, in particular is the risk of serious opportunistic infection associated with generalized suppression of TNF α , a principal mediator of inflammation [5].

Medically, biologic agents such as these offer great promise; the challenge lies in developing methods of delivery that safely and effectively exploit their therapeutic potential. It is in this respect that gene-based approaches offer unique opportunities. This review will summarize the current status of the development of gene-based treatments for chronic joint disease, focusing on those that have the greatest potential for near term clinical application. Remaining research challenges will also be discussed.

SYSTEMIC GENE DELIVERY

For RA, an autoimmune disease that affects joints and other organs throughout the body, several gene-based treatments have been investigated that similarly take a “systemic” approach. These strategies are designed to genetically modify cells in tissues, such as muscle, liver or spleen, which then release the transgene products into the circulation, providing the prospect of inhibiting disease in multiple joints with a single procedure. The types of transgenes selected for evaluation have generally fallen into two categories: those with products that inhibit specific inflammatory cytokines, primarily TNF α and interleukin-1 (IL-1), and those directed toward broad immunomodulation with the goal of down-regulating autoimmune reactivity.

For systemic delivery of TNF and IL-1 inhibitors, a gene-based approach could improve clinical application over administration of recombinant protein. By providing sustained endogenous synthesis of the protein as a transgene product it would eliminate the need for repeat dosing, reducing cost as well as the incidence of adverse responses from injection site reactions [6]. Several methods have been used to test the proof of concept in rodent models,

including intravenous injection of recombinant adenovirus, as well as intramuscular injection of several viral and non-viral vector systems. In animal models, anti-arthritis effects have been observed from systemic delivery of soluble type I and II TNF receptors [7–10] as well as IL-1 receptor antagonist (IL-1Ra) [11], IL-1 receptor accessory protein [12] and soluble type II IL-1 receptor [13].

It is in these systemic treatment approaches that non-viral gene delivery has shown the greatest promise for chronic articular disease. Although plasmid-based transgene expression is typically transient in joint tissues [14], within the post-mitotic muscle cells, plasmid-based systems are considerably more stable [11]. Additionally, the efficiency of plasmid delivery and transgene expression in muscle can be enhanced significantly by electroporation techniques, such that reasonably persistent, anti-arthritis levels of transgene expression have been reported [7, 8, 15].

Perhaps the most comprehensive study concerning systemic gene therapy for RA performed to date was recently described by Sandalon *et al.* In this work, an adeno-associated virus (AAV) vector containing the coding region for a homologous rat soluble TNF α receptor-immunoglobulin Fc fusion, was packaged in AAV serotype 1 (AAV1) capsid [10]. A single intramuscular injection of this vector into the quadriceps muscles of both legs of rats resulted in serum levels of the TNF inhibitor exceeding 30 μ g/ml for over a year, but more importantly showed long term (>150 days) protection from recurrent arthritic episodes using a streptococcal cell wall-induced model.

From a practical standpoint systemic gene delivery of targeted inflammatory cytokine inhibitors would provide a clear delivery advantage over recombinant protein, requiring much less frequent administration. However, this approach still faces the problem of eliciting side effects from systemic inflammatory suppression, such as increased risk of tuberculosis, opportunistic infection, lymphoma and demyelinating diseases, among others [6, 16]. Moreover, feasibility has only been demonstrated in small rodents. Maintaining functional levels of transgene product (1–10 (μ g/ml) in the blood volume of a human would require a level of transgene expression several log orders greater. Achieving this would likely require administration of very high doses of recombinant virus. Concerning non-viral gene transfer, given the increased mass of the human muscle relative to rodents, it is uncertain how electroporation might be scaled upward; multiple sequential delivery events would likely be required.

Given the risk of adverse response, any clinical exploration of these systemic methods will almost certainly require technologies that effectively regulate transgene expression, enabling transcription only in the presence of a specific inducing agent. Toward this direction, Gould *et al.* have shown initial demonstration of regulated systemic expression *in vivo* following intramuscular delivery of plasmid containing a doxycycline-regulated promoter driving a dimeric soluble TNF receptor [8, 17]. Although systems such as this may provide some level of control over transcription, in the event of infection or other adverse event they cannot readily diminish the pre-existing levels of protein product already in the circulation.

In attempts to inhibit autoimmune reactivity, systemic delivery of cytokine transgene products such as IL-10 [15, 18, 19] IL-4[20, 21], interferon-beta [22, 23] and TGF- β 1 [24] has shown promise in inhibiting the onset of collagen induced arthritis in mice, and even more importantly, therapeutic effects in established disease in this model [18, 20, 25]. Most *in vivo* studies of arthritis gene therapy have involved prophylactic treatment, where the ability of the vector and transgene product to inhibit disease onset is measured. In clinical practice, however, patients seeking treatment will already be symptomatic for several months, if not years. Thus the capacity to reverse an ongoing pathologic condition is an important consideration. In addition to intravenous and intramuscular injections, alternate vector delivery approaches have been examined including intraperitoneal injection of plasmid DNA [24], subcutaneous injection of genetically modified fibroblasts [21]; intradermal plasmid delivery via gene gun [26], and recently an intranasal procedure involving aerosolized delivery of plasmid encoding IL-10 was reported to inhibit disease onset as well as progression [25].

These types of studies have provided important information regarding the *in vivo* activity of certain regulatory cytokines in modulating inflammation and autoimmunity. However, from a clinical standpoint, safety concerns regarding generalized immunosuppression will in all likelihood limit the exploration of systemic gene delivery approaches to animal models. More so than the targeted anticytokine approaches mentioned above, proteins such as IL-4 and IL-10 have broad immunomodulatory effects that are neither antigen nor disease specific [27, 28]. Sustained systemic release of these agents could limit the recipient's capacity to respond to infection, as well as tumor formation or could further disrupt self-tolerance. Moreover, pleiotropic agents, such as TGF- β 1 have the capacity to activate numerous diverse cellular pathways that can lead to pathologic effects. Indeed, systemic overproduction of TGF- β 1 is responsible for inducing pathologic renal and pulmonary fibrosis in scleroderma [29, 30]. In our opinion, for non-fatal arthritic conditions, the level of risk associated with systemic gene delivery of broad-spectrum immunomodulatory agents is prohibitive to their serious consideration for clinical study.

LOCAL, INTRA-ARTICULAR GENE DELIVERY

By far the most research progress to date regarding gene therapy for joint disease has been generated in local applications. In general, these strategies involve genetic modification of the cells and tissues specifically within afflicted joints with the goal of changing the biology of the chronically diseased tissues. It is in this direction that gene based strategies offer their greatest advantages, providing opportunities for treatment that cannot be duplicated by conventional methods of drug delivery. Gene therapy for joint diseases was originally conceived as a sophisticated protein delivery system whereby the cells that populate the connective tissues within and encapsulating the joint would be genetically modified to continually produce and secrete large quantities of anti-arthritic proteins [31]. The diseased joint would, thereby, continually manufacture and release its own medicine providing effective lasting treatments for chronic diseases.

There are several key advantages to a local approach. Foremost, it concentrates the therapeutic transgene product specifically where it is needed, within the joint at the site of

disease. Further, by containing transgene expression within the joint, it limits exposure of non-affected tissues and prevents systemic immunosuppression. The local approach could also enable the use of gene products that only occur or function intracellularly such as certain transcription factors, intracellular signaling molecules and siRNAs.

The architecture of the joint lends itself very favorably to local gene-based strategies. For diarthrodial joints, such as the knee, the articulating regions of the bones are surrounded by a complex network of tendons and ligaments that provide support and lateral stability. These dense, fibrous tissues form a capsule that encloses and defines the joint space, which is filled with a limited volume of synovial fluid that lubricates the articulating surfaces. The joint capsule is lined with synovium, a thin vascular, highly cellular tissue. Following injection into the joint space recombinant vectors and genetically modified cells are largely contained locally; the fibroblastic cells that populate the synovium and fibrous tissues are highly receptive to genetic modification, and the dense collagenous matrix of the capsule typically delimits the diffusion of vector from the joint to non-articular tissues. Fibroblastic cells injected into the joint space readily attach and colonize the lining [32]. While there is some movement of proteins both entering and leaving the joint capsule, this semi-closed system permits an overall accumulation of transgene products within the synovial fluid which diffuses through the cartilage and capsular tissues.

Investigation of local gene therapies has for the most part followed two parallel tracks: 1) the evaluation of candidate gene products for *efficacy* in models of disease, and 2) evaluation of methods and vectors for *efficiency* of gene delivery to articular tissues.

Identification of gene products with anti-arthritic properties has been quite successful. A comprehensive list is shown in Table 1, and displays the wide assortment of diverse transgenes that have been reported to inhibit disease onset or progression following local delivery in animal models of arthritis. As with systemic approaches, gene products that specifically target the activity of TNF α and IL-1 have been tested most frequently. Unfortunately though, very few studies have been conducted that compare anti-arthritic transgenes on a head to head basis using the same vector and disease context. Thus, from the perspective of efficacy, there is little information available in the literature that definitively distinguishes any one transgene over another.

In our opinion, once evidence of efficacy is clearly established, the most important consideration when choosing a candidate transgene for clinical study is its safety. For this reason, we have worked most often with the cDNA for IL-1Ra, and used it in the first clinical trial of a gene therapy for arthritis [33]. The protein product serves as a competitive inhibitor of the type I IL-1 receptor and has no agonist or stimulatory activity. It also has an excellent safety profile clinically when used in its recombinant form, anakinra.

Much of the discussion in the previous sections has focused on gene delivery as a platform for sustained production of secreted anti-inflammatory or immunomodulatory agents. A central feature of pathogenesis in RA is the expansion and activation of the synovial lining. Much like an aggressive tumor, following chronic inflammatory stimulation the synovium dramatically expands and becomes hypercellular. The activated tissue then invades and

erodes the cartilage and bone. Similar to those used in cancer, several investigators have explored local gene-based approaches to treatment that are intended to block the expansion of the synovial tissue or to selectively target it for ablation. These approaches have included intra-articular delivery of cDNAs for angiostatin [34, 35] and endostatin [36], p53 [37], Fas ligand [38] and TRAIL [39]. For the ablative methods, sustained transgene expression is not an issue; the only requirement is a high efficiency of delivery such that a large proportion of the cells express the cytotoxic/apoptotic transgene product. Each of the strategies shown in the table has reported some success, and the use of HSV thymidine kinase gene in an ablative approach [40] was approved for clinical study, but this has since been abandoned. From a pre-clinical perspective these approaches are effective, but their movement into clinical study has been hindered by concerns of toxicity in extra-articular tissues.

Ex Vivo Methods

Genetically modified synovial fibroblasts, dermal fibroblasts and bone marrow-derived mesenchymal stem cells have been evaluated for use in ex vivo gene transfer to joints. Synovial fibroblasts retrovirally transduced to express IL-1Ra were initially explored [41], and following promising results in animal models were examined in two clinical trials with no adverse effects [42]. Overall, each of these cell types is amenable to gene transfer and perform similarly following intra-articular injection; the only remarkable difference being the accessibility of the cells, with dermal fibroblasts being the most abundant and readily obtainable. [43]

Regarding ex vivo, intra-articular gene transfer the primary considerations are that the cells are autologous and are modified to express homologous protein gene products, as the joint tissues are highly immune sensitive (see below). Although MSCs have been reported to have immunosuppressive properties [44–46], this has not been our experience with regard to intra-articular gene transfer. We have found that homologous MSCs expressing, foreign, xenogenic transgenes do not persist in the joint, and allogenic MSCs expressing homologous transgenes are likewise eliminated from the joint tissues following ex vivo gene transfer.

Ex vivo gene transfer offers certain advantages over injection of recombinant vectors into the joint space, in that the genetically modified cells can be pre-screened for adventitious agents, levels of transgene expression and potential tumorigenicity. Furthermore, no unbound viral particles or other vector systems are delivered to the recipient. However, significant drawbacks are associated with the time and expense of harvesting tissues from each subject, growing and expanding the cells, genetic modification *in vitro*, and subsequent safety screening of the cells prior to delivery. Moreover, during the clinical trial involving ex vivo delivery of IL-1Ra, we found the growth rate of the primary cells varied widely among subjects as did the receptiveness of the respective cultures to genetic modification [42]. Fibroblasts from some subjects required extended culture and several rounds of infection simply to achieve minimum levels of product secretion. Altogether, these considerations cast doubt on the practicality of ex vivo gene delivery approaches and their applicability to chronic joint disease on a widespread scale.

Direct Gene Transfer

A far more economical and efficient approach is to genetically modify the articular cells *in situ* by direct intra-articular injection of recombinant vectors. However, the number and locations of the genetically modified cells are then determined by the interplay of the properties of the vector with the fluids and tissues of the diseased joint. Although direct gene transfer is far more efficient than *ex vivo* delivery, there is a trade-off of control for efficiency.

In general, the cells within the synovium and joint capsule are comprised of fibroblasts and macrophage like cells. The fibroblastic cells are typically amenable to genetic modification with most viral vector systems and will comprise the vast majority of cells modified following intra-articular injection; the macrophage-like cells are largely refractory to genetic modification *in vivo* and do not contribute significantly to the total level of transgenic expression achieved [47]. For the most part, the chondrocytes within the articular cartilage are not physically available for genetic modification following intra-articular injection of vector systems. Although the cells are receptive, the dense extracellular matrix of the tissue effectively excludes most vectors. There are reports of AAV-mediated transduction of chondrocytes in cartilage explant cultures [48], but efficient gene delivery to these cartilage cells *in vivo* has not been rigorously documented.

VECTOR SYSTEMS

Numerous systems both viral and non-viral have been examined for direct gene transfer to articular tissues. In general non-viral gene transfer has not been satisfactory; levels of transgene expression are sub-therapeutic and persist for less than three days. Additionally, plasmid DNA is inflammatory at the high doses required to achieve measurable protein expression [14]. Although there exist isolated reports of effective non-viral gene delivery to articular tissues, the overwhelming consensus is that non-viral technologies are not yet suitably developed to consider for direct gene delivery to joint tissues. Attempts to augment the efficiency of intra-articular gene transfer using electroporation [49] and sonoporation have been reported but have not been widely successful [50]. In contrast, most of the well-developed viral vectors have been found to be capable of efficient gene delivery and provide transgene expression at biologically relevant levels. This includes, adenovirus, adeno-associated virus, herpes simplex virus and lentivirus. Each of these systems, though, has its advantages and limitations.

Adenovirus

Recombinant adenovirus, due to its capacity for highly efficient gene transfer combined with relative ease of construction and propagation has served as the workhorse for research involving intra-articular gene transfer. This system has proven extremely useful for examining the biological activities of candidate gene products both *in vitro* and *in vivo*. Transgene expression onsets quickly, typically within 24 hours, and is robust, generally persisting at meaningful levels in the joints of immunocompetent animals for 7–14 days [51, 52]. This window is sufficient to enable examination of the biological activity of candidate transgenes in animal models of disease. The primary limitation of this system is that the

widely used first generation vectors retain most of the native viral coding regions and generate low level expression of viral proteins in transduced cells. In the joint tissues, this stimulates antigen-specific cellular immunity and leads to targeted elimination of the adenovirally infected cells, thereby abbreviating transgenic expression. Recent work in athymic, T-cell-deficient rats has shown that in the absence of functioning T cells, long term (>6 months) functional transgenic expression is achieved with this vector [47]. This indicates that in the absence of immune recognition, the vector is capable of providing sustained transgenic expression, and importantly that genomic integration is not a requirement for vector persistence intra-articularly (see below). Although high capacity, helper-dependent adenoviral vectors exist that contain no viral coding regions and thus may enable sustained transgene expression in immunocompetent hosts [53], difficulties generating high titer preparations in appreciable quantities have limited experimentation *in vivo* with this system. Recent advances in adeno-associated virus technologies, which make production easier and adenovirus-free, have begun to shift research emphasis away from adenovirus.

Lentivirus

Lentivirus are a subclass of the retroviridae family, and the most common lentiviral vectors are derived from human immunodeficiency virus I (HIV-1) [54]. The primary benefit to lentiviral systems compared to oncogenic retroviral vectors, such as those derived from Moloney Murine Leukemia Virus (MoMLV), is that they are capable of infecting non dividing cells and thus can be used for *in vivo* gene transfer [55]. Similar to MoMLV, most lentiviral vector systems integrate their genetic material into the genome of the target cell. The transduced cell is permanently modified, and the integrated vector is passed on to all cell progeny [54]. Concerning chronic joint diseases, which likely will require long-term production of therapeutic agents, this potential stability could be advantageous. In joint tissues of animal models, such as rabbits and rats, VSV-G pseudotyped [56], HIV-1 based lentivirus was found to be very efficient, generating exceptionally high levels of transgenic expression [57, 58]. Interestingly, when delivered to the joints of naïve animals that were subsequently stimulated with interleukin-1, transgenic expression was enhanced 6–10 fold [57]. This was found to be due to the proliferation of the stably transduced articular cells in response to the inflammatory cytokine. In diseases, such as RA, this type of response could be beneficial in that the cells that produce the anti-arthritis gene product would be amplified to produce increased levels of gene product during inflammatory flares.

An additional advantage to the use of lentivirus is that the vector does not contain viral coding sequences, reducing the immunogenicity of the transduced cell. Indeed, in the joints of immunocompetent rats, dose dependent, long-term expression was achieved at functional levels following lentiviral-mediated delivery of a homologous soluble TNF receptor [47]. Thus overall, lentiviral vectors possess many attributes that would favor their use in treatment of chronic joint conditions. Regarding clinical application, recent development of lentiviral vectors from species, such as cats [59] and horses [60], may allay the stigma associated with the use of a vector derived from HIV-1. However, concerns regarding the safety of lentiviral vectors and their capacity for random integration and the frequency of insertional mutagenesis remain unresolved. Until these key issues are settled the use of

lentiviral vectors in gene therapy will likely be limited to fatal conditions and experimental animals.

Adeno-Associated Virus

In efforts to identify gene transfer technologies that are both immune compatible and suitable for application in humans, interest in AAV has increased dramatically over the last several years. Along these lines, recombinant AAV has many properties that favor its use as a gene delivery vehicle for the treatment of chronic joint diseases. The wild type virus is not associated with any human disease. The vector form does not contain native viral coding sequences and does not integrate into the genome of the target cell with significant frequency. Also, the vector can be cross-packaged in a variety of AAV capsid serotypes and variants providing the opportunity for enhanced transduction efficiency, and in turn reduced viral load. Importantly, these alternate vector capsids provide the potential to evade pre-existing neutralizing antibodies generated from humoral immune response to natural infection with wild AAV or prior treatment with AAV-derived vectors [61–63].

Compared to other viral systems AAV is relatively simple from a structural standpoint, consisting of a ~5kb single-stranded DNA genome packaged in a small, non-enveloped particle by three nearly identical capsid proteins (viral proteins; VPs1–3) [64]. The only required DNA *cis*- elements are 145 nucleotide inverted terminal repeats (ITRs) that form unique hairpins [63]. While the simple composition of the vector offers potential advantages with regard to safety and immune evasion, it can be an impediment to transduction. This multi-step process involves attachment of the virus to specific cell-surface receptors, endocytosis and intracellular transport in early and late endosomes, endosomal escape, entry into the nucleus and uncoating, and finally the conversion of the single DNA strand into a duplex form that can then be recognized by the nuclear transcriptional proteins [65]. Other than a phospholipase A2 motif found on VP1 of AAV2, which is thought to aid in endosomal escape [66], the vector particle does not contain proteins that actively mediate or facilitate transduction. Following receptor binding, AAV infection is passive, and is directly dependent upon the endogenous biological activity of the specific target cell. Because of this, the efficiency of AAV-mediated gene transfer is widely variable among species, tissues and cell types.

Unlike adenovirus, which can be propagated in most molecular biology laboratories, AAV is relatively difficult to prepare at high titer, requiring trained staff and dedicated facilities. Thus, due to limited access to this vector, the utility of AAV as a vehicle for gene delivery to articular cells and joint tissues, has not been rigorously investigated, and the limited reports in the literature are largely inconsistent in their findings. Several studies, however, have reported beneficial effects following local AAV-mediated gene delivery in experimental arthritis in mice and rats. Overexpression of cDNAs for soluble TNF receptors [67–69], beta interferon (IFN-beta) [70], and angiostatin [35] has been reported to inhibit the onset of joint pathology.

Although these model systems provide an indication of the capacity of the transgene product to inhibit the pathology induced by the disease model, the beneficial results reported are not strongly indicative of the utility of AAV for transduction of joint tissues. Unfortunately the

inflammatory pathology in rodent models such as collagen-induced arthritis and streptococcal-wall induced arthritis, occurs almost exclusively in the ankles and paws. These arthrodial, or gliding joints, are extraordinarily small and architecturally complex without a readily identifiable joint space and, thus, cannot be reliably targeted for intra-articular injection. Furthermore the small rodent ankle cannot physically contain the 25–50 μ l fluid volume [35, 67–73] of virus suspension typically injected. As such, the viral particles are not delivered specifically within the joint but are injected periarticularly, and the viral suspension bathes all the tissues of the lower limb and paw. Interestingly several studies from independent laboratories indicate that murine synovial fibroblasts are inherently resistant to transduction with conventional AAV based vectors. Indeed, work by Cottard *et al.* indicates that the primary site of AAV transduction following injection in the ankle region is extra-articular muscle [72].

With regard to the AAV serotype considered most appropriate for use in joint tissues, this too remains unresolved and without clear consensus. A report by Apparailly *et al.* indicates a preference for AAV5 over AAV2 in the knee joints of mice [74]. However in cultured human synovial fibroblasts, one study reports that AAV2 and 5 are similarly efficient [71], while another indicates the following order of preference: AAV2 > 1 > 5 > 8 [75]. Of further relevance to human application, Bessis *et al.* recently showed that in synovial fluid samples collected from a limited number of RA patients, about half contained neutralizing antibody to AAV1 and 2 capsid serotypes [75]. However, the relationship between intra-articular transgene expression and the immunity that arises from pre-existing infection with natural wild type AAV, or from prior delivery of AAV vectors, remains unknown. Despite this, Targeted Genetics Inc. has initiated a phase I/II clinical trial involving the use of AAV2 for the delivery of the gene equivalent of etanercept to the joints of subjects with RA, and the majority of subjects participating in this trial either have, or will receive repeat injections of the vector. Unfortunately, since measurement of transgene product synthesis is not performed as part of this study, information regarding the utility of AAV as a vehicle for gene delivery to human joints or the capacity to effectively redose AAV in humans will not be forthcoming in the near future. Clearly, intra-articular immunity to AAV and its evasion by alternate serotypes is an area that merits in depth investigation.

In our laboratory, we have found the conventional single-stranded AAV vector to be significantly less effective than HSV, adenovirus or lentivirus for gene delivery to articular cells in culture or *in vivo*. In the knee joints of rats, rabbits and horses, the onset of transgenic expression is significantly slower, requiring at least one to two weeks, and the resulting levels of expression are low and typically sub-therapeutic. Several groups have shown that certain stimuli, such as UV radiation which increases the production of endogenous DNA repair and synthesis proteins, can significantly enhance intra-articular transgene expression from conventional AAV vectors [76–78]. This indicates that second strand DNA synthesis is rate-limiting in AAV transduction of joint fibroblasts. Accordingly, we have found that AAV vectors that are self-complementary(sc) (i.e. double stranded, containing both + and – DNA strands) provide >20-fold increase of transgene expression with a rapid onset in synovial and capsular cells *in vitro* and *in vivo* in the joints of experimental animals. scAAV-mediated transgene expression profiles in the knees of rabbits

were temporally similar to those achieved with adenovirus and with levels sufficient to mediate therapeutic responses in these animals.

scAAV vectors can be produced either by generation of vector plasmids that are ~half-genome sized combined with selective purification of the infectious double stranded form [79], or through the use of half-genome sized vector plasmids containing a mutation in one of the terminal resolution sequences of the AAV ITRs [80]. Both strategies generate + and – strand viral genomes that are covalently linked at one terminal repeat. Because the AAV genomes of the vector are half wild-type size (~2.5 kb) the resulting 2x viral construct (~5 kb) can be packaged into the normal AAV capsid. This modification of the AAV vector overcomes the limitations imposed by poor second strand DNA synthesis that occurs in many cell types.

Despite recent advances in technology, the biology of AAV transduction in articular cells remains poorly understood and largely enigmatic. Although self-complementary vectors significantly augment transduction efficiency, substantial barriers to transduction appear to remain at the level of intracellular trafficking. Currently, very high numbers of viral particles are necessary to achieve transduction of human, rat and rabbit synovial fibroblasts, generally in the range of 10^4 – 10^5 viral particles per cell; even then only 10–20% of AAV genomes reach the nucleus. Work by Jennings *et al.* has shown that the addition of proteasome inhibitors, such as carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (zLLL), dramatically enhances nuclear uptake of AAV genomes in human synovial fibroblasts and is accompanied by a proportional increase in transgenic expression [81]. Similar enhancement of transgene expression was observed in the presence of high levels of inflammatory cytokines; this effect was blocked by addition of wortmannin, a phosphatidylinositol 3-kinase inhibitor, to the culture medium. The phosphatidylinositol 3-kinase pathway is an important contributor to vesicular trafficking and facilitates functional rearrangement of the cytoskeleton required for nuclear targeting of AAV [82]. Altogether, these data indicate that intracellular trafficking is an important barrier to transduction in articular cells and may be linked to either the ubiquitin-proteasome system or to intracellular movement of virus or virus-containing vesicles to nuclear pores.

GENE DELIVERY AND THE BIOLOGY OF THE JOINT TISSUE

Having demonstrated conclusively that transgenes can be delivered to joint tissues and the encoded products expressed at functional, therapeutically relevant levels, an important area that needs to be addressed is the biology of the joint tissues with regard to sustained expression. As mentioned previously the vast majority of studies have focused on evaluating gene products in *short-term efficacy* studies as well as vector systems for their *efficiency* of gene delivery. However, with respect to strategies based on long-term inhibition of chronic disease, a key aspect that has been largely overlooked is the *duration or persistence* of transgenic expression. Several early studies reported sustained intra-articular transgene expression, but used *lacZ* as a marker gene; however, we have since found much of this data to be unreliable. First, the joint tissues are exquisitely immune sensitive, and the cytotoxic T cells of immunocompetent animals will eliminate cells in joint tissues expressing non-homologous transgene products, such as *bacterial* β -galactosidase, within 2–3 weeks [47].

Second, there are numerous endogenous activities in articular tissues capable of cleaving the x-gal substrate thus, generating false positives. These activities are amplified many fold in the inflamed and activated tissues of the arthritic joint. Other indirect assays for transgene expression, such as immunohistochemistry are also highly prone to false positives and can generate misleading, artifactual results.

We have recently performed a series of focused studies designed specifically to determine the capacity of cells in the joint capsule to maintain expression of exogenous transgenes over time. In this work, we initially used T-cell deficient rats to examine transgene expression in the absence of cell-mediated immunity that could lead to premature termination [47]. Following intra-articular injection of recombinant lentivirus or adenovirus containing the cDNA for human IL-1Ra, as a quantifiable secreted transgene product, we found that expression was initially high, but dropped 60–70% within about 6 weeks of vector delivery. After this point, IL-1Ra production leveled off and persisted for the remaining life of the animals. This pattern was identical for both the integrating lentivirus and the episomal adenovirus and did not rely on promoter origin; persistence of expression from the cytomegalovirus (CMV) promoter/enhancer and eukaryotic translation elongation factor 1 α (EF1 α) promoter sequences was indistinguishable. Using GFP as a cytologic marker (Fig. 1), we found that two sub-populations of cells contributed to the overall pattern of expression: 1) transient cells with a half-life of about 2–3 weeks, and 2) stable cells that remained in the tissues indefinitely post-modification. Surprisingly, we found the transient subpopulation of cells was comprised almost exclusively of synovial fibroblasts. The cells capable of long-term expression were found primarily in the fibrous tissues of the capsule, the tendons and ligaments. In healthy immunocompetent rats, we found this very same pattern of expression, but only with the use of a homologous transgene, a rat soluble TNF receptor, delivered by a lentivirus that does not contain viral coding regions. We believe these studies are important, because they show that the biology of the cells and joint tissues can support a gene based treatment platform based on sustained expression of a therapeutic transgene. They also, however, dramatically underscore the importance of immune compatibility of both vector and transgene. Altogether, these results indicate that once the issue of immune compatibility is overcome the natural behavior of the cells is the most critical factor in determining the persistence of transgenic expression, perhaps more so than the specific gene transfer technology.

While intra-articular gene therapy was originally intended to target synovial fibroblasts these results suggest they are not the best cell type upon which to base a treatment platform based on long-term expression [32]. The success of the therapy, however, does not depend specifically on these cells, but simply requires genetic modification of cells within the joint that are long-lived and whose secreted products can reach diseased tissues either by diffusion through the synovial fluid or release into neighboring tissues.

These results illustrate the importance of having a clear understanding of the biology of the joint as a target tissue for genetic modification. Indeed, patterns of cellular transduction *in vivo* are determined by the biophysical interaction of the vector with the specific target tissue- its extracellular matrix, the resident cell populations and their density, the vascularity, local extracellular fluids, etc. This being the case, in RA the phenotype of the articular tissue

is dramatically different from that of the normal joint. The synovium, which in healthy joints is only a few cell layers deep, becomes massively thickened, fibrotic, and hypercellular from the local proliferation of fibroblasts and macrophage, and the infiltration of inflammatory cells. The cells within the expanded tissues become activated and form a pannus that invades and erodes the cartilage and subchondral bone. The synovial fluid similarly increases in volume, viscosity and enzymatic activity. This milieu changes dramatically the landscape into which the vector is delivered and the availability of the cells to the virus.

With very few exceptions *in vivo* studies of local arthritis gene therapy have been conducted using a prophylactic treatment mode where the vector is administered either prior or simultaneously to the onset of disease. This is understandable, since the models of arthritis typically used, such as collagen-induced arthritis in the mouse, are severe with sudden and dramatic onset. The intensity of disease is such that it is extraordinarily difficult to reverse once established. Thus, the vast majority of *in vivo* studies of local gene delivery to joints have involved gene delivery into essentially normal joint tissues. However, in clinical application patients seeking treatment will already have disease, and very little is known mechanistically regarding gene delivery into the arthritic joint. The relative receptiveness to infection, and the stability of the cells under disease conditions has never been examined directly. Because RA is a primary disease target for gene-based treatment, a much-needed area of research is to determine how the changes in tissue morphology associated with disease may affect patterns of transgene delivery and expression, locally and extra-articularly.

Furthermore, a critical but under-recognized consequence of local gene-based treatment for RA, is the capacity of the transgene product to reverse the articular pathology, and in turn the potential for this to adversely impact transgene expression patterns. For example, if the vector when injected into an RA joint preferentially transduces fibroblasts of the intimal layer, transgene expression would be high initially. However, if the expressed protein product is effective and decreases the level of synovitis, these transduced cells will also be lost as the cellularity of the hypertrophied tissue is reduced. Thus, in the RA environment the capacity of the vector to traverse the pannus or synovitis to infect and transduce stable cell populations is an important functional consideration.

CONCLUDING REMARKS

Considerable progress has been made following the initial proof of concept of intra-articular gene delivery. Numerous cDNAs have been found to be effective in ameliorating the pathogenic effects of experimental models of articular disease, and the benefits and limitations of the more characterized gene delivery systems have largely been established. Further, recent developments have shown the capacity of certain cells types in articular tissues to support long term, persistent expression of exogenous transgenes, thus providing cause for realistic optimism regarding the development of effective gene-based approaches for the treatment of chronic joint diseases. There remain however several technical challenges before this can be realized.

During the preparation of this manuscript, a young woman participating in a phase I/II gene therapy trial for RA, involving AAV2 mediated delivery of the cDNA for the gene equivalent of etanercept, died soon after receiving a second intra-articular injection of the vector. The results of an in depth investigation point to the cause of death as a massive retroperitoneal hematoma associated with systemic histoplasmosis. Analysis of post-mortem tissues showed that the vector genomes were not found at significant levels beyond the injected joint. Furthermore, the levels of TNF inhibitor in the circulation were within the expected range for a patient receiving treatment with adalimumab. These findings indicate that the experimental gene therapy treatments did not directly contribute to the death. Rather, the generalized immunosuppression arising from systemic administration of adalimumab appears to be responsible.

Recently the FDA lifted the clinical hold on the trial, allowing it to proceed in a modified fashion. Vector cannot be given to subjects with a temperature greater than 98.6°F or with unexplained fatigue or malaise on day of administration. Patients with a history of opportunistic infection are excluded, and patients are required to have failed at least one disease modifying anti-rheumatic drug (DMARD). Subjects who will receive a second injection of vector will first sign a revised informed consent. The FDA is also requiring additional monitoring, including blood draws at additional time-points after administration of vector for complete blood count, serum chemistry, vector DNA, TNFR:Fc protein and potential T-cell responses to AAV2 capsid.

Although this tragic event appears to be unrelated to the gene delivery procedure, its investigation has dramatized how little is genuinely understood regarding AAV biology and the fundamental mechanics of intra-articular gene transfer. It underscores the need for further research to understand more clearly the potential risks, especially those associated with long-term expression of therapeutic gene products and their effects on the local and systemic biology of the recipient. Further, the development of cellular and humoral immune responses, against both the vector and transduced cells and their effects on vector readministration remain largely unexplored, but are critical to effective clinical application. Indeed, the more we understand about the targeted delivery of specific gene transfer technologies the more informed judgements can be made regarding their safety, utility and the applications for which they may or may not be suitable.

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LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AdHKBP	Adenovirus containing the human kallistatin binding protein
AIA	Antigen-induced arthritis
cDNA	Complementary DNA

CIA	Collagen-induced arthritis
CIS3	Cytokine-inducible SH2-containing protein
CMV	Cytomegalovirus
CTLA-4 Ig	Cytotoxic T-lymphocyte antigen 4 immuno globulin fusion protein
DMARD	Disease modifying anti-rheumatic drug
EF1 α	Elongation factor 1 alpha
FasL	Fas ligand
GFP	Green fluorescent protein
HSP70	Heat shock protein 70
HSV TK plus ganciclovir	Herpes simplex virus thymidine kinase plus ganciclovir
HSV	Herpes simplex virus
IFNβ	Interferon β
IGF-1	Insulin like growth factor 1
IKKβ-DN	Dominant negative inhibitor of kappa β kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-1Ra	Interleukin 1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-4	Interleukin-4
ITR	Inverted terminal repeats
LPS	Lipopolysaccharide
MSC	Mesenchymal stem cells
NF-κB decoy	nuclear factor-kappa B decoy
ODN	Oligodeoxynucleotide
Ras-DN	Ras-dominant negative
sc	Self-complementary
SCW	Streptococcal cell wall
sIL-1R	Soluble interleukin-1 receptor
siRNA	Small interfering ribonucleic acid
SOD	Superoxide dismutase

srIkBa	Super-repressor inhibitor kappa B alpha
sTNFR	Soluble tumor necrosis factor receptor
TGF-β1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
TNFR:Fc	TNF receptor extracellular domains fused to the Fc domain of IgG
TRAIL	TNF related apoptosis inducing ligand
UV	Ultra violet
VIL-10	Viral interleukin-10
VP	Viral proteins
VSV-G	Vesicular stomatitis virus G protein
zLLL	Carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal

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Fig. (1).

Changes with time in the location and density of transduced cells following intra-articular gene transfer. Lentivirus or adenovirus containing the cDNA for GFP was injected into the knee joints of athymic nude rats. At various timepoints the animals were killed and the joint tissues were analyzed by fluorescence microscopy (A). As shown in the series of panels at Day 5 large numbers of GFP+ cells were seen throughout the synovial lining and in the surrounding capsular tissues. By Day 168 the vast majority of the GFP+ cells in the synovial lining had disappeared. In contrast, the number of GFP+ cells in the fibrous capsular tissues,

ligaments and tendons, as well as the interstitial cells in muscle remained largely unchanged. Comprehensive tabulation of the locations of GFP positive cells is shown in panel B.

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Table 1

Transgenes and Vectors Used to Inhibit Experimental Arthritis

	Transgene*	Method**	Vector***	Animal Model	Reference
Targeted Cytokine Inhibitors	IL-1Ra	Ex Vivo	Retrovirus	Rabbit AIA	[Otani <i>et al.</i> 1996; 41]
				Rat SCW-induced arthritis	[Makarov <i>et al.</i> 1996; 83]
	(Canine)			Mouse zymosan, CIA	[Bakker <i>et al.</i> 1997; 84]
				Dog ACL transaction (OA)	[Pelletier <i>et al.</i> 1997; 85]
				Rabbit meniscectomy (OA)	[Zhang <i>et al.</i> 2004; 86]
			Adenovirus	Mouse IL-1Ra KO	[Hur <i>et al.</i> 2006; 87]
				Horse osteochondral defect (OA)	[Frisbie <i>et al.</i> 2002; 88]
	(Equine)	<i>In Vivo</i>	HSV	Rabbit IL-1 overexpression	[Oligino <i>et al.</i> 1999; 89]
			AAV	Rat LPS-induced arthritis	[Pan <i>et al.</i> 2000; 90]
			Lentivirus	Rabbit IL-1 overexpression	[Gouze <i>et al.</i> 2003; 57]
			Plasmid	Rabbit meniscectomy (OA)	[Fernandes <i>et al.</i> 1999; 91]
	IL-1RAcP	Ex Vivo	Stable transfection	Mouse CIA	[Smeets <i>et al.</i> 2003; 12]
	sIL-1R	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Ghivizzani <i>et al.</i> 1998; 51]
			Retrovirus	Rabbit AIA	[Kim <i>et al.</i> 2002; 92]
	TNFR:Fc	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Ghivizzani <i>et al.</i> 1998; 51]
Immuno Modulatory Proteins			AAV	Rat SCW-induced arthritis	[Chan <i>et al.</i> 2002; 68]
				Rat adjuvant-induced arthritis	[Adriaansen <i>et al.</i> 2007; 67]
	sTNFR	<i>In Vivo</i>	AAV	TNF-transgenic mouse	[Zhang <i>et al.</i> 2000; 93]
			Plasmid	Mouse CIA	[Gould <i>et al.</i> 2004; 17]
				Rat adjuvant-induced arthritis	[Woods <i>et al.</i> 2001; 94]
	IL-4	<i>In Vivo</i>	Adenovirus	Mouse CIA	[Geurts <i>et al.</i> 2007; 95]
				Rat adjuvant-induced arthritis	[Haas <i>et al.</i> 2006; 96]
	IL-10	<i>In Vivo</i>	Retrovirus	Mouse CIA	[Tamer <i>et al.</i> 2002; 97]
				Rabbit meniscectomy (OA)	[Zhang <i>et al.</i> 2004; 86]
	vIL-10	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Lechman <i>et al.</i> 2003; 98]

	Transgene*	Method**	Vector***	Animal Model	Reference
				Rabbit AIA	[Lechman <i>et al.</i> 1999; 99]
		Ex Vivo	Plasmid	Mouse CIA	[Kuroda <i>et al.</i> 2006; 100]
	IL-13	<i>In Vivo</i>	Adenovirus	Rat adjuvant-induced arthritis	[Woods <i>et al.</i> 2002; 101]
	IFN β	<i>In Vivo</i>	AAV	Rat adjuvant-induced arthritis	[Adriaansen <i>et al.</i> 2007; 102]
			Adenovirus	Rat adjuvant-induced arthritis	[Adriaansen <i>et al.</i> 2006; 70]
	CTLA-4 Ig	<i>In Vivo</i>	Adenovirus	Mouse CIA, AIA	[Ijima <i>et al.</i> 2001; 103]
	Angiostatin	<i>In Vivo</i>	Lentivirus	Mouse CIA	[Kim <i>et al.</i> 2002; 92]
	Endostatin	<i>In Vivo</i>	Lentivirus	TNF-transgenic mice	[Yin <i>et al.</i> 2002; 36]
	FasL	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Yao <i>et al.</i> 2000; 38]
				SCID Mouse and Rabbit	[Zhang <i>et al.</i> 2005; 104]
Anti Angiogenic and Synovial Ablators	HSV TK plus ganciclovir	<i>In Vivo</i>	Adenovirus	Monkey CIA	[Goossens <i>et al.</i> 1999; 105]
	Pl6	<i>In Vivo</i>	Adenovirus	Rat SWC-induced arthritis	[Taniguchi <i>et al.</i> 1999; 106]
	p21	<i>In Vivo</i>	Adenovirus	Rat adjuvant-induced arthritis	[Nonomura <i>et al.</i> 2001; 107]
	p53	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Yao <i>et al.</i> 2001; 37]
	Ras-DN	<i>In Vivo</i>	Adenovirus	Rat adjuvant-induced arthritis	[Yamamoto <i>et al.</i> 2003; 108]
	TRAIL	<i>In Vivo</i>	Adenovirus	Rabbit AIA	[Yao <i>et al.</i> 2003; 39]
	AdHKBP	<i>In Vivo</i>	Adenovirus	Rat AIA	[Wang <i>et al.</i> 2005; 109]
	Catalase	<i>In Vivo</i>	Stable transfection	Rat AIA	[Dai <i>et al.</i> 2003; 110]
	CIS3	<i>In Vivo</i>	Adenovirus	Mouse CIA, AIA	[Shouda <i>et al.</i> 2001; 111]
	HSP70	<i>In Vivo</i>	Plasmid	Rat (OA)	[Grossin <i>et al.</i> 2006; 112]
Others	IGF-1	<i>In Vivo</i>	AAV	Rats CIA	[Izal <i>et al.</i> 2007; 113]
	IKKp-DN	<i>In Vivo</i>	Adenovirus	Rat adjuvant-induced arthritis	[Tak <i>et al.</i> 2001; 114]
	NF- κ B decoy	<i>In Vivo</i>	ODN/liposome	Rat CIA	[Tomita <i>et al.</i> 1999; 115]
	srI κ B α	<i>In Vivo</i>	Adenovirus	Rat SWC-induced arthritis	[Miagkov <i>et al.</i> 1998; 116]
	SOD	<i>In Vivo</i>	Stable transfection	Rat AIA	[Dai <i>et al.</i> 2003; 110]
	Prothymosin- α	<i>In Vivo</i>	Adenovirus	Rats CIA	[Shiau <i>et al.</i> 2007; 117]
	Thrombospondin 1	<i>In Vivo</i>	Adenovirus	Rat CIA	[Jou <i>et al.</i> 2005; 118]

* IL-1Ra= IL-1 receptor antagonist; IL-1RAcP= IL-1 receptor accessory protein; sIL-1R= soluble IL-1 receptor; TNFR:Fc=TNFR extracellular domains fused to the FC domain of IgG; sTNFR= soluble tumor necrosis factor receptor; IL-4=interleukin-4; IL-10=interleukin-10; VIL-10=viral IL-10; IL-13=interleukin 13; IFN β =interferon β ; CTLA-4 Ig=CTLA-4 Ig fusion protein; FasL= Fas Ligand; HSV TK

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plus ganciclovir=herpes simplex virus thymidine kinase plus ganciclovir; Ras-DN=Ras-dominant negative; TRAIL=TNF related apoptosis inducing ligand; AdHKBP=Adenovirus containing the human kallistatin gene; CIS3=cytokine-inducible SH2-containing protein; HSP70=Heat shock protein 70; IGF-I=insulin like growth factor I; IKK β -DN=IKK β -dominant negative; NF- κ B decoy=nuclear factor-kappa B; srlkB α =super-repressor I κ B α ; SOD=superoxide dismutase

** HSV=herpes simplex virus; AAV=adeno-associated virus; ODN=oligodeoxynucleotide

*** SCW=streptococcal cell wall; CIA=collagen-induced arthritis; LPS=lipopolysaccharide; AIA=antigen-induced arthritis;