Review

Gene therapy as a therapeutic approach for the treatment of rheumatoid arthritis: innovative vectors and therapeutic genes

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In recent years, significant progress has been made in the treatment of rheumatoid arthritis (RA). In addition to conventional therapy, novel biologicals targeting tumour necrosis factor-alpha have successfully entered the clinic. However, the majority of the patients still has some actively inflamed joints and some patients suffer from side-effects associated with the high systemic dosages needed to achieve therapeutic levels in the joints. In addition, due to of the short half-life of these proteins there is a need for continuous, multiple injections of the recombinant protein. An alternative approach might be the use of gene transfer to deliver therapeutic genes locally at the site of inflammation. Several viral and non-viral vectors are being used in animal models of RA. The first gene therapy trials for RA have already entered the clinic. New vectors inducing long-term and regulated gene expression in specific tissue are under development, resulting in more efficient gene transfer, for example by using distinct serotypes of viral vectors such as adeno-associated virus.

This review gives an overview of some promising vectors used in RA research. Furthermore, several therapeutic genes are discussed that could be used for gene therapy in RA patients.

Rheumatoid arthritis (RA) is a systemic disease mainly affecting the joints, characterized by chronic inflammation and synovial hyperplasia leading to the destruction of cartilage and bone. RA is associated with a lowered life expectancy and decreased quality of life. The initiating stimulus is still unidentified, but extensive research over the last two decades has clarified the main immunological reactions involved in synovial inflammation and joint destruction [1, 2]. These recent advances in understanding the pathophysiology of RA have enabled the development of new therapeutic targets, allowing disease modification by targeting pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) [3]. Antibodies against TNF-α and soluble TNF-α receptors have proven effective in modulating disease activity and are now used in the clinic.

In general, recombinant proteins are not given orally because of digestion by gastrointestinal enzymes and alternative systemic routes of administration are invasive for the patient. Additionally, these proteins often have a limited half-life, requiring repeated and expensive high dosages to achieve therapeutic concentrations in the joints. These high circulating levels of biologicals can result in serious side-effects. Taken together, there is a clear need for a more efficient delivery system.

Approaches to gene transfer

An attractive alternative for systemic protein delivery is local gene therapy. In this approach, the therapeutic proteins are synthesized within the affected joint. Stable intra-articular (i.a.) synthesis of the therapeutic proteins should allow sustained, local suppression of inflammation while minimizing systemic side-effects. Two approaches for delivering genes coding for a therapeutic protein are used, namely ex vivo or in vivo gene transfer.

Ex vivo or in vivo gene transfer

An ex vivo approach involves the transduction of cells in culture followed by the injection of these modified cells into the target tissue. The genetically modified cells secrete the desired protein at the site of injection. An advantage of this approach is the possibility of using non-viral vectors. Although this method results in a relatively low transfection efficacy, untransduced cells can easily be selected out. Furthermore, cells can be well characterized and expanded before transplantation. The cells used for ex vivo gene therapy should be readily accessible and able to survive for longer periods of time in vitro without significantly changing their phenotype. Primary, autologous cell cultures have a decreased risk of malignant transformation and diminished antigenicity compared with established cell lines. Modified T cells [4, 5], dendritic cells [6, 7] and fibroblasts [8] (delivered systemically), as well as myoblasts (delivered locally) [9], have proven useful in gene transfer in several animal models of RA. Primary skin fibroblasts and fibroblast-like synoviocytes (FLS) may be most promising. They are relatively easy to obtain and to reinfuse. Furthermore, the fact that they are actively involved in the destructive processes seen in RA makes them attractive targets.
for therapeutic intervention [10, 11]. Clonal expansion of a selected phenotype has been found not to interfere with normal functioning of FLS [12]. Most studies make use of adenovirus (Ad) as a vector, encoding viral proteins, thus limiting the duration of expression. Recent experiments have shown the possibility of stable transfection of FLS and skin fibroblasts with artificial chromosomes \textit{in vitro}. Subsequent i.a. injection in rats with adjuvant arthritis (AA) resulted in expression of the marker gene in the joint [13]. Using this expression system, no viral vectors are needed and these artificial chromosomes have, in contrast to viral vectors, an enormous capacity for combining multiple synergistic therapeutic gene cassettes and regulatory promoters [14].

A second strategy, \textit{in vivo} gene transfer, involves the direct delivery of genes, usually by viral vectors, to target tissue. The \textit{in vivo} approach allows production of the desired protein by the resident cells, without the risk of \textit{in vitro} modification of the cells. This can be achieved by one injection, with minimal manipulation of the target cells. \textit{In vivo} gene therapy has been studied with varying success in various animal models of RA using recombinant Ad, recombinant adenoassociated virus (rAAV) and lentiviral vectors [15–19].

\textbf{Route of administration}

Transduced cells or viral vectors can be delivered systemically or locally. Systemic injection will result in relatively high circulating levels of therapeutic protein and lower concentrations in the joints. In this way, all joints can be reached by a single injection containing the vector. Good results have been obtained in mice, using viral vectors or naked DNA. Using electroporation, plasmids coding for IL-10 [20] and soluble p75 TNF receptor–Fc fusion protein [21] successfully transduced muscle, and suppressed collagen-induced arthritis (CIA) in mice. Intramuscular injection of rAAV expressing genes such as IL-4 [22] and IL-10 [23] resulted in long-term expression of the therapeutic proteins and prevented (IL-4) or reduced (IL-10) murine collagen-induced arthritis (CIA). Although RA is a systemic disease, it has clearly localized manifestations, arguing for local treatment to avoid the side-effects of systemic delivery. For gene therapy purposes in particular, RA fits the criteria for successful local \textit{in vivo} gene transfer. The joint space is a relatively closed compartment, minimizing shedding of the vector and allowing transduction of cells only at the site of inflammation. The i.a. injection to reach the synovium is relatively simple and non-invasive. Resident cells, such as FLS, have a low mitotic rate ensuring long-term expression of the transgene [24].

An illustration of \textit{in vivo} gene therapy for RA is shown in Fig. 1. However, a critical requirement for clinical success is the choice of the right vector.

\textbf{Vectors for intra-articular gene transfer}

Several systems have been studied for the delivery of nucleic acids to cells. Although some success has been reported \textit{in vivo} with naked DNA using electroporation or liposomes, persistent expression is limited to muscle tissue. In contrast, viruses have evolved to be highly efficient in stable transduction of various cell types. Great progress has been made in the last decade in manipulating the viral genome and deleting genes coding for viral proteins. In this way, pathogenicity is limited while gene transfer capacity has improved. In the next section, the vectors commonly used for gene therapy in RA are discussed.

\textbf{Adenoviral-mediated gene transfer in RA}

Ad vectors are able to transduce dividing and non-dividing cells and are widely used for gene delivery \textit{in vitro} and \textit{in vivo}. \textit{In vivo} transduction with first-generation Ad results in a very high

\textbf{FIG. 1. Local \textit{in vivo} gene therapy for RA.} rAAV is injected into the arthritic joint by intra-articular injection. Cells present in the synovium (FLS, macrophages, dendritic cells, B cells and T cells) can be transduced and will produce the therapeutic protein at the site of inflammation.
Transduction efficacy in a variety of cell types, but the expression is transient due to immunogenicity. Adenoviruses are non-enveloped DNA viruses. The genome consists of about 36 kb of double-stranded linear DNA [25]. The first generation recombinant Ads were generated by deleting the E1 genes, which encode proteins that are essential for replication and viral gene expression [26]. E1-deleted Ads, however, express low levels of viral proteins after infection. This inevitably induces immune responses directed against the transduced cells, leading to a rapid loss of therapeutic protein expression [27, 28].

Therefore, recombinant Ads have been developed by deleting not only the E1 gene, but also E2 and E4 genes [29]. The immunogenicity of such vectors is reduced, but unfortunately so is the duration of the protein expression, probably due to transregulation of expression [30]. Despite the availability of third and fourth generations, having E1/E2a/E3/E4 deleted [31], most studies in animal models of RA have been performed with the first and second generation of these vectors.

Human synoviocytes are efficiently transduced by Ad in vitro [32], and numerous animal studies showed synovial expression of therapeutic proteins (IL-10 [33, 34], IL-1Ra [35], human 55 kDa TNF-α receptor [36], IL-13 [37] and IL-4 [38–41]) after i.a. injection. The duration of expression varies between 2 days and 8 weeks, but in general the expression of the protein is optimal 3–7 days after injection and declines rapidly thereafter. There seems to be an optimal curve with respect to the dosage injected, as shown in mice [42], rats [15], rabbits [43] and rhesus monkeys [32]. Although once discovered as potential vectors for gene therapy, the currently used Ads are too immunogenic, resulting in a duration of expression that is too short for therapeutic expression of proteins in a chronic disease such as RA. However, this approach can be useful in delivering apoptosis-inducing (e.g. TRAIL) genes to the proliferating synovium, with partial synovectomy as a result. Any transduced cells left are then rapidly cleared from the system to prevent unwanted apoptosis [44]. Recombinant Ad can be produced to high titres and remains a powerful tool for screening new therapeutic genes for their usefulness in animal models of rheumatic diseases.

The most recent generation of recombinant Ad comprises ‘gutted’ vectors, which have all viral genes deleted. These vectors only contain the inverted terminal repeat (ITR) required for replication and packaging [45, 46]. Production of such vectors requires the use of a helper virus to provide the viral proteins in trans [25]. Recent improvements in production techniques [47] and promising results with respect to transduction of retina [45], muscle cells [48–50] and liver [51] indicate the great promise of these new vectors for future testing in animal models of arthritis.

rAAV-mediated gene transfer in RA

Originally discovered as contamination in batches of Ad, adenovirus-associated virus (AAV) is now widely used in gene therapy research. AAV is a small, non-enveloped single-stranded DNA virus in the family of human parvoviruses and is non-pathogenic and non-toxic in humans. After infection, AAV remains latent until the cell is superinfected with a helper virus, such as Ad or herpes simplex virus. AAV is then rescued from this latent stage and uses these helper viruses to replicate. The main advantage of rAAV, which is unable to replicate, is that the absence of viral genes prohibits a host immune response thereby allowing stable gene transfer and long-term expression of the transgene. Recently, cross-packaging of serotypes allowed the transduction of a wide variety of tissues and cell types. In addition, its physicochemical stability permits easy storage and administration. In the past, the major drawbacks were cumbersome production and purification techniques, but new techniques have been developed to overcome these limitations [52–57].

To produce rAAV, the replicase (rep) and capsid (cap) open reading frames, coding for non-structural and capsid proteins, respectively, are deleted to keep only the terminal repeats necessary for replication and integration of the virus. Wild-type AAV (WIAAV) can stably integrate into a specific locus on chromosome 19 (AAVS1 on q13.3-qter) of dividing and non-dividing human cells [58, 59]. Integration of rAAV, however, is random, and circular forms can exist episomally as well [60]. Both mechanisms will result in long-term transgene expression in different cell types of several species [61]. A concern, however, is the possibility that rAAV may integrate in transcriptionally active regions.

At present rAAV serotype 2 (rAAV2) is being used in clinical trials in haemophilia B [62, 63], leucodystrophy [64], cystic fibrosis [65, 66], RA [67] and other conditions. No adverse events due to the vector have been observed and long-term protein expression has been achieved. In animal models of RA, all rAAV studies so far have been done using serotype 2. Most cells found in the joint can be transduced in vitro by rAAV2, including human chondrocytes [68–70], human and murine FLS [71], human macrophages (JA, unpublished data) and human cartilage [69]. In vivo, expression has been shown to last for up to 200 days and was detected in synoviocytes, chondrocytes and myocytes [72–74].

In a rat model for RA, rAAV2.LacZ was injected into both healthy and lipopolysaccharide (LPS)-induced arthritic joints [73]. After 3 days 95% of the synovial lining cells in arthritic joints decreased to 70% in 14 days and to 8% after 30 days. Of interest, diminished transgene expression could be efficiently reactivated to 95% positive cells after 30 days by a repeated LPS boost [73]. In contrast, healthy joints showed minimal staining. A similar experiment was performed in the human TNF-α transgenic (TG) murine model [71]. The majority of transduced cells was FLS, but chondrocytes and meniscal cells were transduced by rAAV2 as well. rAAV2.LacZ was also tested in the CIA model [71]. Expression was reported for up to 7 months in the arthritic joints, with intense staining in the suprapatellar pouch and popliteal bursa [74]. This study, however, was later dismissed as a possible artefact. Studies delivering the rAAV2 vector intramuscularly demonstrated transgene expression, detected in serum and muscle, for up to 4 months [23, 75]. Together, these studies show long-term expression after gene delivery using rAAV in animal models. Studies investigating the delivery of genes using rAAV2 in models of RA are summarized in Table 1.

In recent years, other naturally occurring AAV serotypes have been discovered as well, of which eight are well characterized. Although they are very similar in genetic structure and biological properties, the capsid has less than 50% homology in amino acid sequence. This not only creates unique epitopes for the immune system to recognize, but also makes it possible for rAAV to interact with different receptors on target cells. As a consequence, all serotypes display a different tropism. To study these differences in the virion shells, transgenes utilizing the AAV2 ITRs are packaged into the different serotype specific virions. This results in cross-packaged rAAV vectors, only differing in the virion capsid proteins responsible for entering the target cells [79–81]. An example of cross-packaging is schematically shown in Fig. 2.

It was found, for example, that rAAV1 was superior in transducing muscle [81], rAAV4 was most efficient in infecting retina [82], rAAV5 in nervous tissue [83, 84], airway epithelia [85, 86] and retina [87] and rAAV8 was best in transducing liver [88, 89]. Recently, we have compared the efficacy of five cross-packaged rAAV vectors using the capsid of AAV serotype 1–5, in transducing synovial tissue after i.a. injection in both AA in rats and CIA in mice [76, 78]. rAAV5 was more competent in transducing arthritic synovial tissue after i.a. injection in arthritic rats and mice compared with rAAV1-4. These differences in transduction profiles are probably related to distinct mechanisms
of uptake and subsequent intracellular unpackaging and trafficking of the virus.

As will be discussed later, the high prevalence of pre-existing antibodies against rAAV2 as well as the antibodies associated with re-administration could also be circumvented by using different AAV serotypes [90, 91]. Thus, switching of rAAV serotype may allow a more efficient, tissue specific, in vivo transduction.

Humoral immune responses to viral vectors

Although several studies have demonstrated successful gene transfer and even partial corrections of function in pre-clinical and phase I studies, activation of the immune response to the vector has occurred.

Transduction of cells in vivo inevitably leads to the presentation of viral antigens by antigen-presenting cells to naïve B cells in the lymph nodes. This results in CD4+ T-cell activation and subsequent differentiation of these B cells into plasma cells, which produce antibodies specific for the viral capsid proteins that may have neutralizing effects. These neutralizing antibodies (NAB) are able to neutralize an infection in vivo by activating the complement system or by binding to viral surface proteins of transduced cells, inducing a specific immune response leading to the clearance of these cells. As a result, in vivo transduction may be complicated by pre-existing immunity directed against WtAd or AAV. Adenoviral infections occur all over the world and specific NAB are detectable in 97% of the population. For Ad type 5, most commonly used for gene therapy purposes, the incidence of pre-existing immunity...
is 50% [92]. Successful circumvention of this pre-existing immunity has been described using alternative serotypes [93]. Promising results have also been obtained in masking the capsid protein by conjugating the vector to polyethylene glycol, thus neutralizing the effects of the humoral response [94].

Similar to immunity to Ad, pre-existing infection with AAV2 is also common, with a prevalence of circulating NAB varying from 35 to 80%, depending on age and geographical location [90, 92, 95]. The effects of these NAB are controversial. Some studies show neutralizing effects in liver [95], lung [86] and brain [91], while others demonstrated no influence on transduction efficacy in lung [96] and muscle tissue [97]. This might have to do with the different mechanisms of entry of rAAV in different cell types. Importantly, in human synovial fluid anti-AAV2 NAB were detectable and anti-AAV2 IgGs were present in all patients tested with a variety of arthritides. In vitro, chondrocyte infection by rAAV2 was inhibited by these NABs. The neutralizing capacity of synovial fluid was correlated with the level of NAB present [98].

Antibodies to AAV2 are most common and potent, followed by NAB against serotypes 1 and 3. Anti-AAV5 antibodies are less widespread, with a prevalence varying from no detectable levels of NAB up to 13% (monospecific) [90] in a population of 85 healthy individuals [99]. Moreover, endogenous sequences of AAV5, suggesting previous infection with this serotype, could not be detected in a large diverse group of individuals of different sexes, races and ages [100].

Considering rAAV gene therapy as a local therapeutic approach, the level of NAB against the serotype used should be minimal, with limited cross-reactivity between NAB against different serotypes, allowing readministration. Conceivably, NAB against the capsid of the initial rAAV serotype used for treatment might hinder a second infection with a different serotype. The effects of readministration on transgene expression have been extensively studied in different animal models, but the results are not conclusive. An overview of these studies is summarized in Table 2.

### Table 2. Overview on transduction efficacy after in vivo re-administration of rAAV serotypes

<table>
<thead>
<tr>
<th>Target tissue</th>
<th>Animal</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>Transgene expression after second injection</th>
<th>NAB after first injection</th>
<th>Time of second injection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>1</td>
<td>1</td>
<td></td>
<td>No transduction</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>2</td>
<td>1</td>
<td></td>
<td>Reduced 3-fold</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>2</td>
<td>2</td>
<td></td>
<td>No transduction</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Lung</td>
<td>Murine/rabbit</td>
<td>2</td>
<td>2</td>
<td></td>
<td>Reduced 20-fold</td>
<td>Yes</td>
<td>21 days</td>
<td>86, 102</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>2</td>
<td>2</td>
<td></td>
<td>No transduction</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Lung</td>
<td>Rabbit</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Successful</td>
<td>Yes, promoter dependent</td>
<td>21 days, 3rd at week 17</td>
<td>96</td>
</tr>
<tr>
<td>CNS</td>
<td>Rat</td>
<td>2</td>
<td>2</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>30 days</td>
<td>103</td>
</tr>
<tr>
<td>Muscle</td>
<td>Murine</td>
<td>1</td>
<td>2</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Muscle</td>
<td>Murine</td>
<td>5</td>
<td>2</td>
<td></td>
<td>Successful</td>
<td>n.d.</td>
<td>28 days</td>
<td>99</td>
</tr>
<tr>
<td>Lung</td>
<td>Murine</td>
<td>6</td>
<td>2</td>
<td></td>
<td>Successful</td>
<td>Yes, not much</td>
<td>21 days</td>
<td>86</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>1</td>
<td>2</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>30 days</td>
<td>101</td>
</tr>
<tr>
<td>Lung</td>
<td>Rabbit</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>Successful</td>
<td>Yes</td>
<td>17 weeks</td>
<td>96</td>
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<tr>
<td>Muscle</td>
<td>Murine</td>
<td>5</td>
<td>5</td>
<td></td>
<td>No transduction</td>
<td>n.d.</td>
<td>28 days</td>
<td>99</td>
</tr>
<tr>
<td>Lung</td>
<td>Murine</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Successful</td>
<td>n.d.</td>
<td>5 months</td>
<td>104</td>
</tr>
<tr>
<td>CNS</td>
<td>Rat</td>
<td>2</td>
<td>5</td>
<td></td>
<td>Successful</td>
<td>Yes, promoter dependent</td>
<td>&gt;14 days</td>
<td>103</td>
</tr>
<tr>
<td>Muscle</td>
<td>Murine</td>
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<td>5</td>
<td></td>
<td>Successful</td>
<td>n.d.</td>
<td>28 days</td>
<td>99</td>
</tr>
<tr>
<td>Lung</td>
<td>Murine</td>
<td>6</td>
<td>6</td>
<td></td>
<td>Reduced</td>
<td>Yes, not much</td>
<td>21 days</td>
<td>86</td>
</tr>
<tr>
<td>Lung</td>
<td>Murine</td>
<td>2</td>
<td>6</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>21 days</td>
<td>86</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>8</td>
<td>8</td>
<td></td>
<td>No transduction</td>
<td>Yes</td>
<td>56 days</td>
<td>105</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>1</td>
<td>8</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>56 days</td>
<td>105</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>2</td>
<td>8</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>56 days</td>
<td>105</td>
</tr>
<tr>
<td>Liver</td>
<td>Murine</td>
<td>7</td>
<td>8</td>
<td></td>
<td>Successful</td>
<td>Yes</td>
<td>56 days</td>
<td>105</td>
</tr>
</tbody>
</table>

NAB, neutralizing antibodies; CNS, central nervous system; n.d., not detected.

The effects of readministration on transgene expression have been extensively studied in different animal models, but the results are not conclusive. An overview of these studies is summarized in Table 2.

### Tissue- and disease-inducible promoters

One way to circumvent unwanted ectopic transgene expression is the use of tissue-specific promoters. A transgene driven by a promoter responding only to tissue-specific transcription factors will theoretically only be expressed in the target tissue. Examples of tissue-specific promoters are: kallikrein promoter for salivary gland [106], albumin gene for liver [107], glial fibrillar acidic protein promoter for astrocytes [108] and collagen II promoter for joint chondrocytes [109].

Certain promoters can also be used to control transgene expression, following the intermittent course of disease. Due to the chronic and relapsing nature of RA, therapeutic gene expression should be maximal during flare-ups of the disease and should be switched off during remission of the disease. In general, two systems exist: control by pharmacological systems or by physiological stimuli. Using pharmacological systems, gene expression is controlled by oral antibiotics. The most widely used pharmacologically regulated system is the tetracycline (Tet)-off [110] and Tet-on [111] expression system, expressing in the absence or presence of tetracycline or its derivative doxycycline (Dox) supplementation, respectively. The application of this system in vivo is promising with no immunogenicity in mouse and primate models [112, 113]. Viral IL-10 was successfully delivered to muscle using a Tet-on promoter in a rAAV2 vector in murine CIA [23]. This resulted in a significant reduction of arthritis in immunized and boosted mice treated with Dox. In the same model, a human...
dimeric soluble TNF-α receptor (dsTNFR) expressed from plasmid DNA in a constitutive [cytomegalovirus (CMV) promoter] or regulated manner (Tet-on promoter), was delivered intramuscularly by electroporation [114]. Inhibition of disease activity by dsTNFR was shown in mice with mild arthritis. In mice receiving the Dox-driven dsTNFR plasmid this effect was dependent on oral supplementation of Dox. This effect was, however, not potent enough to cause improvement in mice suffering from more severe arthritis. This might be caused by the route of administration (intramuscular delivery rather than i.a.).

An alternative to this pharmacological approach is to create a self-regulating system by using physiological characteristics of the disease to control transgene expression. Regulatory elements of pro-inflammatory cytokines or transcription factors that are up-regulated during active disease can be used to control the expression of the therapeutic gene. Encouraging results have been obtained in a study combining the human IL-1 enhancer sequence in front of the human IL-6 promoter [115]. In contrast to the commonly used CMV promoter, showing an initial high activity which declines rapidly, this promoter has low basal activity and high activity during the inflammatory response. This was demonstrated by constructing an Ad containing the promoter in front of the luciferase gene. Fibroblasts and macrophages transduced in vitro expressed luciferase in response to inflammatory signals. In vivo the i.a. expression could be reactivated in mice by streptococcal cell wall (SCW) injection [115].

Another innovative expression system responds to the transcription factor nuclear factor (NF)-κB with amplification transcription. This vector, C3-Tat/HIV, consists of a complement 3 (C3) promoter depending on NF-κB activation and driving the expression of a truncated HIV Tat molecule that retains transactivator activity. Tat in turn induces expression of the therapeutic gene on the same vector [116, 117]. In two studies, this system was used to express hIL-1Ra or hIL-10 using an adenoviral vector. In rat [118] as well as mouse [119] models of arthritis transgene expression was regulated by disease activity, resulting in diminished disease activity and decreased paw swelling. It should be noted, however, that Tat is an immunogenic protein, possibly causing an immunogenic reaction or having adverse effects by transactivating host genes [120].

**Therapeutic targets for gene therapy**

**Pro-inflammatory cytokine inhibition**

The number of possible therapeutic genes potentially useful for gene therapy of arthritis is still growing. Several molecules may be used simultaneously, either by using gene therapy or by combining gene therapy with systemic biological therapy. TNF-α plays a key role in the inflammation and joint damage that occur in RA [121]. TNF-α controls in part the production of IL-1 and other pro-inflammatory cytokines, including IL-6 and IL-8. Furthermore, it increases the expression of adhesion molecules, chemokines, prostaglandin E2 and matrix metalloproteinases (MMPs). TNF-α is found at high levels in the synovial fluid of patients with RA, and in synovial tissue its expression is strongly correlated to disease activity [122].

The importance of TNF-α in RA is demonstrated by several experimental and clinical observations [121, 123, 124]. TNF-α blockade after the onset of disease resulted in amelioration of clinical symptoms and prevention of joint destruction. Recently, it has been shown that local expression of an anti-TNF single-chain antibody blocked disease development in mice with CIA [125] and lowered levels of inflammatory Th1-driven IgG2a antibodies to CII [126]. Antagonizing the actions of both IL-1 and TNF-α using gene therapy approaches in several animal models of arthritis showed prevention of disease progression and joint destruction [75, 114, 119, 127–129]. The combination of TNF blockade and IL-1 blockade by adenosival gene therapy in arthritic rabbits is even more effective than blockade of either cytokine alone [130]; this synergistic effect, however, was not observed in humans.

In humans, biologicals targeting cytokines and their receptors have been proven useful as specific therapies for RA [131, 132]. Drugs that block the activity of TNF-α have been shown to improve clinical symptoms in RA patients. Infliximab and adalimumab, both antibodies against TNF-α, and etanercept, a fusion protein of TNF receptor II, are used in the clinic to treat RA. Because of the complex pathology, combination therapy is usually necessary and the most effective therapy utilizes both TNF-α blockade and methotrexate treatment. It is now possible to obtain 20% improvement in about 60–70% of the RA patients using this approach. The majority of these ACR 20% responders, however, will still have some actively inflamed joints. In addition, despite the promising results, these systemic therapies are not devoid of side-effects [133]. The occurrence of infections, most importantly reactivation of tuberculosis in patients receiving TNF-neutralizing agents, is for instance considerably higher than in the general population, and discontinuing therapy may cause severe relapse of the arthritis [134]. These side-effects are in part due to the fact that treatment is administered systemically. Development of local gene therapy targeting TNF might provide a novel method to treat arthritis at the site of inflammation, which is especially relevant in case of isolated arthritis activity.

IL-1 has also been proven to be a critical cytokine involved in synovitis in RA [135]. Endogeneous production of IL-1 receptor antagonist (IL-1Ra) is an important anti-inflammatory mechanism, both in animal models and in human disease. In the RA synovium, an imbalance in this system exists because the relative levels of production of IL-1Ra are not adequate to effectively block the pro-inflammatory effects of IL-1 [136]. In vitro data suggest that a biological response can occur when only 2–3% of the IL-1 receptors (IL-1Rs) are occupied. Thus, up to 98% of all IL-1Rs needs to be occupied by IL-1Ra before an antagonistic effect is observed.

In experimental animal models, IL-1 inhibition using IL-1Ra has been proven to be an effective strategy [137–139]. The transfer of the IL-1Ra gene provided a far more potent biological effect than administration of the recombinant protein in animal models. An example is provided by the treatment of antigen-induced arthritis in rabbits [140]. Repeated injections of recombinant human IL-1Ra had no effect in this model of RA beyond inhibition of synovial fibrosis, occurring in the chronic stage of the disease. In contrast, a dramatic beneficial effect was observed on cartilage matrix metabolism as well as a moderate anti-inflammatory effect when IL-1Ra was administered locally to joints via ex vivo gene transfer [43]. Similarly, human IL-1Ra gene transfer into synovial fibroblasts prevented cartilage destruction in human cartilage co-implanted with transduced synovial fibroblasts in severe combined immunodeficiency (SCID) mice [141]. More recently, the efficacy of the soluble form of the IL-1 receptor accessory protein (sIL-1RaC) was demonstrated as an inhibitor of IL-1 in CIA [142, 143]. sIL-1RaC was over-expressed both locally (knee joint) and systemically in CIA in mice using either an adenoviral vector encoding sIL-1RaC or a stable-transfected NIH3T3 fibroblast cell line. From these and other experimental arthritis models, it has become clear that administration of IL-1Ra, both locally and systemically, has a protective effect on cartilage and bone turnover and reduces the inflammatory cell infiltration normally seen in experimental arthritis.

IL-1Ra has been tested in clinical trials and anakinra, the recombinant form of IL-1Ra, has been approved for clinical use in the treatment of RA [144, 145]. Anakinra treatment mainly protects against joint destruction and is marginally anti-inflammatory. Limitations of IL-1Ra as a pharmaceutical
compound include its lack of oral availability and its short biological half-life. However, a phase I trial in RA patients has recently demonstrated that human IL-1Ra cDNA can be safely transferred to and expressed within rheumatoid joints using \textit{ex vivo} gene therapy [146]. An additional advantage of the delivery of IL-1 inhibitors via gene therapy is that in this way a molecule is produced that has been subjected to authentic post-translational processing. The recombinant molecule lacks glycosylation and since it has an extra amino-terminal methionine, the molecule produced \textit{in vivo} after gene transfer may have more biological potency.

\textbf{Over-expression of anti-inflammatory cytokines}

Anti-inflammatory cytokines are also of potential interest because they can suppress and counterbalance Th1-driven responses and inhibit production of pro-inflammatory cytokines. Over-expression of anti-inflammatory cytokines showing a therapeutic effect in animal models of RA include interferon (IFN)-\(\beta\), IL-4, IL-10 and IL-13 [18, 19]. We will focus here on the potential of IFN-\(\beta\) as a therapeutic cytokine for use in gene therapeutic approaches. IFN-\(\beta\) is a cytokine with pleiotropic effects and is expressed in rheumatoid synovial tissue [147]. Based on \textit{in vitro} work and experiments in animal models of RA, the effects are mainly anti-inflammatory. Of special interest is the ability of IFN-\(\beta\) to reduce the secretion of TNF-\(\alpha\), IL-1\(\beta\) and IL-6, which are all key players in the pathogenesis of RA. At the same time IFN-\(\beta\) could enhance the production of the anti-inflammatory mediators IL-1Ra and IL-10.

More recently, it was shown that IFN-\(\beta\) is important in maintaining homeostasis of bone resorption [148, 149]. Bone remodelling depends on a delicate balance between bone formation and bone resorption, wherein bone-forming osteoblasts and bone-resorbing osteoclasts play central roles. Shifting the balance in favour of osteoclasts leads to pathological bone resorption as seen in RA and osteoporosis [149].

Treatment of CIA in mice and monkeys using daily IFN-\(\beta\) injections resulted in clinical improvement, decreased synovial inflammation and protection against joint destruction [150, 151]. Similar data were obtained after systemic IFN-\(\beta\) gene therapy in murine CIA [8]. However, treatment of RA patients with IFN-\(\beta\) has been unsuccessful so far, presumably as a result of pharmacokinetic issues [152]. Recently, the potential of i.a. gene therapy using an adenoviral vector to determine whether local constitutive expression of IFN-\(\beta\) might have a beneficial effect was studied in rats with AA [153]. It was demonstrated that \textit{in vivo} adenoviral gene transfer of IFN-\(\beta\) cDNA to the synovium reduced arthritis activity. In addition, i.a. IFN-\(\beta\) gene therapy protected against joint destruction. In the future, this approach could be beneficial in RA patients using a vector more suitable for long-term expression of the transgene.

\textbf{Inhibition of NF-\(\kappa\)B activation}

The transcription factor NF-\(\kappa\)B may also be a good target for gene therapy. NF-\(\kappa\)B is highly activated in the synovium of RA patients, and can induce transcription of pro-inflammatory cytokines, adhesion molecules and inducible nitric oxide [154]. NF-\(\kappa\)B activation also appears to be a pivotal factor protecting the synovial cells against apoptosis. Suppression of NF-\(\kappa\)B can markedly enhance apoptosis in the synovium. Phosphorylation of inhibitor of \(\kappa\)B (I\(\kappa\)B) proteins is an important step in NF-\(\kappa\)B activation and is induced by I\(\kappa\)B kinase (IKK), which consists of three subunits: the kinases IKK-\(\alpha\) and IKK-\(\beta\) [155, 156] and the regulatory subunit IKK-\(\gamma\) (NEMO) [157]. IKK activation initiates I\(\kappa\)B degradation, thereby releasing NF-\(\kappa\)B from the cytoplasmic NF-\(\kappa\)B-I\(\kappa\)B complex for nuclear translocation.

The i.a. injection of decoy oligonucleotides for NF-\(\kappa\)B inhibited the severity of murine CIA [158]. Consistent with these data, the use of a potent NF-\(\kappa\)B inhibitor, SP100030, resulted in a significant decrease in joint swelling in mice with CIA [159]. Clinical efficacy was accompanied by diminished NF-\(\kappa\)B activation as shown by electrophoretic mobility-shift assay (EMSA). Together, these studies show the potential of blocking NF-\(\kappa\)B activity to treat arthritis. This should preferably be done by local treatment in the light of possible unwanted effects of systemic treatment. This could be achieved using different approaches. First, \textit{in vitro} studies using a specific NF-\(\kappa\)B blocking peptide, the NEMO binding domain (NBD) peptide, have demonstrated efficient inhibition of NF-\(\kappa\)B activation in various cell types [160, 161]. The NBD peptide, a cell-permeable peptide that consists of the IKK-\(\beta\) NBD fused with a membrane translocation sequence, is able to inhibit NF-\(\kappa\)B by prevention of the interaction between NEMO and IKK-\(\beta\) required for activation of IKK-\(\beta\). This peptide significantly reduced the severity of CIA in mice by reducing levels of TNF-\(\alpha\) and IL-1\(\beta\), abrogating joint swelling and reducing joint destruction [162]. In rats with AA, i.a. injection of the NBD peptide resulted in significantly reduced severity of arthritis activity and radiological damage [163].

Another approach to inhibit NF-\(\kappa\)B activity may be over-expression of I\(\kappa\)B. I\(\kappa\)B gene transfer in human synovial tissue culture reduces spontaneous production of pro-inflammatory cytokines without an effect on anti-inflammatory mediators [164]. Moreover, I\(\kappa\)B over-expression inhibited the production of MMP-1 and MMP-3 without affecting tissue inhibitor of MMP (TIMP-1). Blocking NF-\(\kappa\)B through \textit{over-expression} of I\(\kappa\)B by adenoviral gene transfer resulted in induction of apoptosis in the synovium with concomitant clinical improvement in rats with arthritis [165]. In Lewis rats with AA adenoviral dominant negative IKK-\(\beta\) (Ad.IKK\(\beta\)dn) significantly ameliorated the severity of disease as evidenced by decreased paw swelling compared with rats treated with the control vector [166].

Recently, we have shown that rAAV5-mediated IKK\(\beta\)dn gene transfer to the synovium also reduces the severity of arthritis in rats with AA when the treatment was started after the onset of disease (manuscript submitted). Thus, a NF-\(\kappa\)B-directed approach might be appealing as an antirheumatic strategy by means of local gene therapy.

\textbf{Contralateral effect}

It has been reported that i.a. injection of viral vectors containing therapeutic genes not only ameliorates arthritis in the injected joints, but also in the uninjected, contralateral joints. This phenomenon was first described in rabbits after i.a. delivery of Ad coding for the receptors of IL-1\(\beta\) and TNF-\(\alpha\) [127]. The beneficial effects seen in the contralateral joints do not seem to depend on the vector nor are they limited to a specific transgene, as retrovirus expressing murine IL-4 i.a. injected into rats also resulted in a therapeutic effect in the contralateral paws [167]. Although the contralateral therapeutic effect is antigen-specific, the exact mechanism remains unclear [168].

There is some evidence that trafficking leucocytes or fibroblasts are responsible for this effect. Experiments with i.a. delivery of an adenoviral vector containing luciferase as a marker gene resulted in luciferase activity in injected joints and draining lymph nodes, but also in the contralateral joints of rabbits [127]. Because none of the organs showed luciferase activity, this is unlikely to be caused by biodistribution of the virus. Other possible mechanisms could involve systemic levels of the therapeutic protein or an undefined neurological process. In addition, DC can secrete exosomes with effects on contralateral joints [169].
Towards clinical trials in RA

A large number of animal studies have demonstrated proof of concept of viral gene therapy. Many types of viral vectors have been employed in more than 700 clinical trials that have been carried out or are currently in progress. However, only a limited number of trials are in the field of arthritic diseases. \textit{Ex vivo} gene therapy using autologous synovial fibroblasts has previously been studied in a study in RA patients in two phase I trials [170]. In both studies, FLS derived from surgical samples of post-menopausal women were transduced \textit{in vitro} with a retroviral vector containing the gene for IL-1Ra and injected into the metacarpophalangeal joints. The data suggest that transfer and \textit{i.a.} expression of the transgenes have been successfully and safely accomplished. However, at this moment no follow-up clinical studies are being planned, presumably due to the logistical and practical limitations of this approach.

New vectors such as the ‘gutted’ adenoviral vectors are promising for future clinical trials, but at the moment rAAV vectors may have greatest potential as gene therapeutics for the treatment of RA. Therefore, here we will focus on the development of rAAV gene therapy. rAAV vectors are currently under evaluation for treatment of cystic fibrosis, haemophilia and Canavan’s disease [62–65]. A clinical trial has also been proposed for Parkinson’s disease [171] and lipoprotein lipase (LPL) deficiency. Of importance, Targeted Genetics (Seattle, WA, USA) has conducted a phase I dose escalation trial to assess the safety of \textit{i.a.} delivery of rAAV2 containing the human TNF receptor–Fc immunoglobulin fusion gene (tgAAC94). This treatment was well-tolerated and appeared to be safe; a second phase I trial has recently been announced.

Before rAAV-mediated gene therapy for RA can enter the clinic, there are a number of important issues to be solved. First, different rAAV serotypes need to be compared. As described earlier, \textit{in vivo} gene transfer using rAAV5 to the synovium appears to be more efficient than with the other serotypes. Newly discovered serotypes should also be tested for their ability to transduce synovial tissue.

After studies in rodents, vectors should be tested in large animals in order to ensure that they mediate safe and long-term gene expression. The CIA rhesus monkey model is a good pre-clinical model of human RA for testing of efficacy and safety [172]. Furthermore, studies in non-human primates that are naturally infected with AAV can provide important information about the role of NAB, which is necessary for the design of future clinical trials.

In addition, studies on appropriate regulation of therapeutic gene expression will be important. Excessive and uncontrolled production of the therapeutic transgene might theoretically lead to spillover to the circulation that will skew the immune system and cause side-effects. Placing a therapeutic transgene under control of a disease-inducible promoter could reduce the risks of spillover and allow treatment to meet the variable demand during the variable course of arthritis activity. Some possible disease-inducible promoters have been described above. There is a need for research on the efficacy of these promoters in animal models of RA.

It will be important to evaluate rAAV vectors carrying a therapeutic gene in relevant animal models as well. Some potential therapeutic genes have already been tested in animal models of RA, but often using vectors other than rAAV. It needs to be determined if expression levels reached after rAAV transduction are sufficiently high for modulating the disease activity. Finally, manufacturing of a clinical grade vector at high titres is an essential issue. One of the major limitations for the use of rAAV vectors for gene therapy has been the difficulty in producing enough vector to supply a clinical trial. At this moment, more than 20000 roller bottles may be required to generate rAAV by the traditional transfection process to treat 50 patients. A lot of progress has been made during the last few years on vector production and purification [173–175]. Recently, a scalable rAAV producer cell-line grown in serum-free media, which can meet the needs for the manufacture of rAAV gene therapeutics, has been developed [174].

Concluding remarks

Local delivery of therapeutic compounds by gene transfer is a promising approach to treat RA with several potential advantages over systemic forms of targeted therapy. Several strategies to deliver DNA have recently become available and new techniques are constantly emerging.

While the search for new vectors (viral and non-viral) continues, rAAV is one of the most promising for RA gene therapy. Although long-term expression can be achieved using rAAV, the pre-existing immunity to Wt viruses remains an issue to be investigated in more detail. Switching of serotype could circumvent the NAB directed against the capsid of the Wt virus, improving infection by its recombinant counterpart. Since tropism of the serotypes differs, more tissue-specific approaches are possible by choosing serotypes best suitable for \textit{i.a.} use.

In the light of the success of TNF blockade in the clinic, TNF-\(\alpha\) inhibition is one of the most promising targets for future gene therapy in RA. Ideally, a transduced synovial cell will respond to inflammatory signals by expression of molecules inhibiting TNF-\(\alpha\) with high affinity. Combining the therapeutic gene under control of an inflammation responsive promoter using an efficient vector holds great potential for future treatment.

While the therapeutic potential of rAAV-mediated treatment appears promising for RA, additional safety studies involving pharmacokinetics, biodistribution and toxicity will need to be accurately evaluated in animal models. The potential risks of the use of some viral vectors are illustrated by the tragic death of a patient participating in an adenoviral gene therapy trial [176, 177] and the development of leukaemia in X-SCID patients after successful treatment of the disease with retroviral gene therapy [178]. These events underscore the importance of an extensive safety programme as well as the need for a profound understanding of the effects of various viral vectors.

Taken together, published studies have firmly established the scientific basis for further development of gene therapy in RA.

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References

Gene therapy in RA: innovative vectors and therapeutic genes


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