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Chapter 3

Retroviral gene therapy for intestinal inflammation

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submitted for publication
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Introduction

Inflammation is a major feature in a vast number of gastro-intestinal (GI) diseases, such as inflammatory bowel disease, cancer,\(^1\) cystic fibrosis,\(^2\) familial adenomatous polyposis,\(^3\) ischemia/reperfusion,\(^4\) radiation enteritis,\(^5\) Barrett's oesophagus,\(^6\) celiac disease,\(^7\)\(^8\) and Helicobacter pylori infection.\(^7\) For some of these disorders there is no satisfactory therapy. Whereas the inflammatory changes are usually confined to the gut mucosa, most medical therapies make use of the systemic administration of drugs that often have severe side effects. We here propose an alternative approach to the delivery of specific immunomodulatory signals to the gut mucosa, using gene transfer. Gene therapy was initially developed for the treatment of inherited diseases with a single gene defect, but at present most of the approved clinical protocols involve cancer patients.\(^9\) More recently, the possibility of treating inflammatory diseases by delivery of anti-inflammatory or immunomodulatory genes has attracted significant interest.\(^10\) In this review we summarize different strategies for using gene therapy in the treatment of gastro-intestinal (GI) inflammation, focusing on retroviral gene transfer.
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Table 1  Potential transgenes in gastro-intestinal inflammation

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant negative I kappa B kinase (IKK-2) mutant</td>
<td>Inhibition of NF-κB activation</td>
<td>52-54</td>
</tr>
<tr>
<td>Anti-inflammatory cytokines (IL-4, IL-10, TGF-β)</td>
<td>Inhibition of Th1 response and antigen presentation</td>
<td>55, 56</td>
</tr>
<tr>
<td>IL-1 receptor antagonist and soluble TNF receptor</td>
<td>Inhibition of actions of pro-inflammatory cytokines IL-1 and TNF</td>
<td>57, 58</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>Antagonism of effects of IL-12p70</td>
<td>59</td>
</tr>
<tr>
<td>CTLA-4 Ig</td>
<td>Inhibition of costimulation</td>
<td>60, 61</td>
</tr>
<tr>
<td>FasL</td>
<td>Induction of apoptosis</td>
<td>62</td>
</tr>
</tbody>
</table>

Abbreviations: NF: nuclear factor; IL: interleukin; TGF: transforming growth factor; TNF: tumour necrosis factor; CTLA-4: cytotoxic T lymphocyte-associated antigen; Ig: immunoglobulin; FasL: Fas ligand.

Requirements for gene therapy

Gene of interest

Gene therapy is the transfer of genetic material to cells with the aim of offering therapeutic benefit. The gene of interest (transgene) is transferred to the target cell by a delivery vehicle (vector), obtaining selective gene expression at the site of interest. The transgene may encode for therapeutic RNA, such as ribozymes or antisense RNA, or for proteins. GI inflammation is regulated by numerous genes that modify the pattern of gene expression (e.g. transcription factors) of inflammatory cells, regulate antigen presentation, and modulate T cell responses (cytokine secretion), accessory signals from costimulatory molecules, cell recruitment via chemotaxis and cell adhesion, as well as more general downstream effector functions. Examples of potential transgenes are provided in table 1.

Vector

A vector is required to facilitate uptake and expression of the transgene into target cells. Vectors can be non-viral (plasmids and liposomes) or viral (adenovirus, adeno-associated
### Table 2 Vectors for gene transfer

<table>
<thead>
<tr>
<th></th>
<th><strong>Viral</strong></th>
<th><strong>Non-viral</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retro-virus</strong></td>
<td>&lt;8 kb</td>
<td>&gt;20 kb</td>
</tr>
<tr>
<td><strong>Lenti-virus</strong></td>
<td>&lt;10 kb</td>
<td>&gt;20 kb</td>
</tr>
<tr>
<td><strong>AdV</strong></td>
<td>&lt;7-8 kb</td>
<td>NA</td>
</tr>
<tr>
<td><strong>AAV</strong></td>
<td>&lt;4 kb</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Liposomes</strong></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td><strong>Plasmid DNA</strong></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td><strong>Integration</strong></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Sustained</strong></td>
<td>Variable</td>
<td>No</td>
</tr>
<tr>
<td><strong>expression</strong></td>
<td>Variable</td>
<td>Transient</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td>Poor</td>
<td>Variable</td>
</tr>
<tr>
<td><strong>delivery</strong></td>
<td>Variable</td>
<td>High</td>
</tr>
<tr>
<td><strong>Quiescent</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>cells</strong></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>Insertional mutagenesis</td>
<td>Cellular toxicity</td>
</tr>
<tr>
<td></td>
<td>Insertional mutagenesis</td>
<td>Local effect only</td>
</tr>
<tr>
<td></td>
<td>Host immune response</td>
<td>Difficult manufacturing</td>
</tr>
</tbody>
</table>

Abbreviations: AdV: Adenovirus; AAV: adeno-associated virus; kb: kilobases; NA: not applicable.

Each vector system is characterized by an inherent set of properties (summarized in table 2) that affect its suitability for specific applications. Any vector should meet four important criteria: safety, efficiency, reliability and preferably inducible expression of the transgene. At present, viral vectors are far more efficient agents for gene transfer than non-viral agents. Viral vectors exploit the natural ability of viruses to introduce their genetic material into the cells they infect with high efficiency.

Treatment of GI inflammation requires additional features of the vector, including long-term local gene expression from a relatively small proportion of cells. A retroviral vector is a promising candidate to accomplish this goal. Firstly, it integrates efficiently into the genome of the target cell. Secondly, it does not transfer any viral gene, thus reducing the risk of an immune response against the transduced cells. Both of these properties are likely to be crucial for achieving sustained expression of the transgene. Lastly, retroviral vectors have a large cloning capacity, up to 8 kb. The distinct disadvantage of traditional retroviral vectors is that the viral particles require disruption of the nuclear membrane in order to gain access to the DNA in the host cell, as a result...
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of which their application is limited to dividing cells such as lymphocytes. Several potential targets of gene therapy are cells that rarely if ever proliferate, such as neurons, hepatocytes, myocytes, crypt cells and macrophages. Unlike the traditional oncoretroviruses, lentiviruses (e.g. the human immunodeficiency virus: HIV) can infect non-mitotic cells, relying on active transport of the so-called pre-integration complex (viral cDNA and viral proteins) through the nucleopore by the nuclear import machinery of the target cell. The most widely accepted theory to explain this property of lentiviruses is that nucleophilic virion proteins chaperone viral nucleic acids across the nuclear envelope.

Clearly, these properties distinguish retroviral vectors from adenoviral vectors. Adenoviral vectors infect a wide number of dividing and non-dividing cells, they are relatively easy to prepare and purify, and the levels of transgene expression are high. A major disadvantage of adenoviral vectors is their episomal status in the nucleus after cell entry, allowing only transient expression of the therapeutic gene. Furthermore, expression of viral genes provokes inflammatory reactions and toxicities that limit the repeated application of adenoviral vectors. Although several modifications of adenoviral vectors may reduce host responses, within the near future the use of these vectors remains restricted to situations that require high levels of gene expression for a limited period of time; examples include cancer therapy, tissue repair and vaccination. For extensive reviews of vectors we refer to recent publications.

Retroviral vectors

Retroviruses are lipid-enveloped particles comprising two identical positive (single-stranded) RNA molecules. The prototype genome consists of three transcriptional units (gag, pol and env) with a long terminal repeat (LTR) sequence (needed for the integration of the genome incorporated in the recombinant retroviral particles into the host genome and containing the retroviral promoter) at both ends. The gag region encodes genes that comprise the capsid proteins; the pol region encodes the reverse transcriptase and integrase proteins; and the env region encodes the proteins needed for receptor recognition and envelope anchoring, thus dictating the host range of the retroviral particles (figure 1). Following cellular entry, the reverse transcriptase synthesizes viral
DNA using the viral RNA as its template. The cellular machinery then synthesizes the complementary DNA, which is circularized and randomly inserted into the host genome. Following insertion, the viral genome is transcribed and viral replication is completed.18

Two varieties of retroviral vectors are used in gene therapy: oncoretroviral vectors (e.g. Moloney Murine Leukemia Virus; MMLV) and lentiviral vectors (e.g. HIV-derived vectors). MMLV-modified viruses are very attractive for clinical gene therapy protocols (table 2).19 These viruses are generated from plasmids containing the gene of interest flanked by the two MMLV LTRs (a retroviral plasmid is shown in figure 2). As no viral genes are present in the plasmid, a packaging cell line, providing all the viral proteins, is required to incorporate the gene of interest within viral particles (figure 3). The modified viral particles are replication-defective and retain only one round of infectivity, since they

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**Figure 1 Structure of the MMLV and HIV retroviral genomes**

A) The proviral MMLV DNA as it is inserted into host DNA is shown, with the long terminal repeats (LTRs) composed of U3, R, and U5 elements at each end. The *gag* (encodes capsid proteins), *pol* (encodes the reverse transcriptase and the integrase) and *env* (encodes the envelope proteins) genes are located invariably in the positions shown in all retroviruses. The (ψ) viral packaging signal directs incorporation of vector RNA into virions.

B) The HIV genome codes for three structural genes (gag, pol and env), common to all retroviruses, and six regulatory genes (Vif, Vpr, Vpu, Tat, Rev, Nef).
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do not contain any viral gene. Because retroviral vectors integrate in the host genome, the transgene is maintained after cell division. Combined with the efficient and sustained expression of the retroviral promoter present in the LTRs (the viral promoter), retroviral vectors have the potential to result in long-term transgene expression.

Safety concerns associated with retroviral vectors include immune responses against cells expressing the therapeutic gene, the potential risk of insertional mutagenesis and contamination with replication competent virus. However, experimental studies have suggested that the frequency of cell transformation after retroviral transduction is not higher than that of spontaneous transformation.

In the early nineties, a clinical study proved the therapeutic benefit of \textit{ex vivo} gene transfer to T cells in patients with adenine deaminase severe combined immunodeficiency. More recently, two patients with severe combined immunodeficiency-X1, a disease with a cytokine receptor \( \gamma \) chain (\( \gamma_c \)) gene deficiency, received retroviral transduced hematopoietic progenitor cells overexpressing the missing gene. After a 10-month follow-up, the disease phenotype was corrected with normal counts of functional, \( \gamma_c \) transgene-expressing T and natural killer cells. To our knowledge, no reports have been published so far on the use of retroviral transduced T cells in experimental or clinical GI inflammation. In other inflammatory disease models such as experimental multiple sclerosis and arthritis, T cells overexpressing anti-inflammatory cytokines such as transforming growth factor (TGF)-\( \beta \) or interleukin (IL)-10 prevented disease.

\textbf{Lentiviral vectors}

Lentiviral vectors are promising vectors for gene therapy, because of the aforementioned unique ability to infect non-dividing cells. In addition to the \textit{gag}, \textit{pol} and \textit{env} genes, lentiviruses have accessory genes such as Vpr and Rev, which complicates the design and production of safe lentiviral vectors (figure 1). The amount of HIV sequence present in the vector is correlated with the risk of generating replication competent virus, and it is still unclear how much of the viral genome can be deleted during vector construction without adversely affecting the efficiency of the vector.

Lentiviral vectors can be pseudotyped with the G protein from the vesicular stomatitis virus (VSV-G) to expand the host range beyond their natural 'target' cells, the
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Figure 2 Structure of a replication defective retroviral vector

The genes necessary for viral replication are replaced by the gene(s) of interest. Efficient gene expression and integration depends on the inclusion in the retroviral vector of a number of viral elements. These include a promoter, a polyadenylation signal, a viral packaging signal \( \Psi \) as well as signals required for reverse transcription such as a transfer RNA-binding site (PBS), a polypurine tract (PPT), a repeated (R) region at both ends of the viral RNA, and short, partially inverted repeats located at the termini of the viral LTRs required for integration.

CD4+ T cells. Significant progress has been made towards the recovery of safe and efficient lentiviral vectors, and the use of these vectors in the development of gene therapy in non-dividing cells represents a reality rather than a promise. There are, however, still problems that will need to be resolved before clinical application will be possible: the lack of a high output, stable packaging cell line for clinical protocols, the frequently observed decline in levels of transgene expression with time and biosafety issues.

Delivery route

The delivery route provides a crude means of the targeting necessary to ensure that the therapeutic gene has the desired effect in the diseased tissue, without being detrimental to healthy tissue. The vector can be delivered to gastro-intestinal tissue in different ways. A straightforward method is the *in vivo* delivery, i.e., the direct introduction of the vector into the patient. An injectable vector should be retained in the organs and tissues that harbor
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Figure 3  Strategy for generation of replication defective retroviral vector

A packaging cell line is generated by stable transfection of all the retroviral genes necessary for replication (gag, pol and env genes). To generate recombinant vector, the retroviral construct containing the gene of interest is transfected into the packaging cells. The expressed viral RNA, by virtue of the packaging signal ψ, is incorporated into maturing viral particles. The supernatants are harvested and used to infect the target cells.

However, in addition to particle binding specificity, other factors such as size, charge, and dispersion will have an influence on vector distribution in the body. For GI
inflammation, *intra-luminal* administration seems a suitable route. For example, peroral administration (through a gastric tube) of an AAV vector expressing β-galactosidase as a marker gene was efficient for lactose intolerance in rats and gave persistent and stable expression in the gastro-intestinal system for up to 6 months. In *vivo* studies showed that intestinal epithelial cell lines could be readily transduced with a lentiviral or adenoviral vector containing a marker gene. Intra-rectal administration of an adenoviral vector containing a cytokine gene in healthy mice induced intestinal but not systemic transgene expression. However, the duration of transgene expression after rectal administration remains to be investigated, as epithelial cell turnover is rapid. Recently a clinical trial was begun in patients with severe Crohn's disease of the rectum, studying subcutaneous and submucosal administration in the rectal and perianal region of the human IL-10 and IL-4 gene complexed to cationic lipids, a non-viral vector. Alternatively, direct *in situ* injection is possible, for example in case of a colorectal tumor accessible via endoscopy.

Adenovirus, AAV, herpes simplex virus or non-viral vectors that can transfer genes to quiescent cells are most suitable for *in vivo* delivery. A disadvantage of *in vivo* administration of gene therapy vectors is the potential exposure of multiple organs to high concentrations of the gene product or the vector, which may lead to side effects. Young and Mautner recently discussed safety issues related to the direct introduction of adenoviral vectors into patients.

Alternatively, gene therapy vectors may be delivered to the target cells outside the patient, and subsequently reinjected, the so-called *ex vivo* delivery route. Although this approach is more time-consuming, and usually needs to be patient-tailored, it does not expose patients to vectors. Moreover, the target cells can be isolated and modified under controlled conditions. *Ex vivo* strategies involving the use of retroviral vectors and peripheral blood mononuclear cells (PBMCs) as target cells, provide an appealing approach to the delivery of genes into the GI tract. For instance, peripheral T cells are easily available, readily expanded in culture and have the ability to migrate to the gastro-intestinal mucosa. The transduction efficiency of primary human T cells approaches 50% or more, using optimized methods of retroviral gene transfer. Furthermore, gene therapy clinical trials using adenosine deaminase or the herpes simplex type 1 thymidine
kinase genes have shown that transduced T cells can persist in vivo for several months up to 4 years.21-31

**Strategies for specific targeting**

Despite all the recent advances in gene delivery, more sophisticated methods of targeting will be needed, since most of the vectors and transgenes used for gene therapy are neither cell nor tissue specific. Targeting viral vectors can be achieved at the level of either gene expression or gene delivery.42-44

In the first place, targeted gene expression (transcriptional targeting) can be accomplished by designing vectors with restricted transgene expression in target cells using upstream genetic elements such as cell-specific promoters, enhancers or silencers. When this approach is used, the integration of the viral vector is not cell-specific, but the transgene is exclusively expressed in cells that have the necessary transcription factor proteins. For example, the 'liver' fatty acid binding protein promotor expressed by the continuously regenerating epithelium could be used to obtain local transgene expression within the intestine.45

Secondly, gene delivery (transductional targeting) can be modified by altering the retroviral vector host range.45, 46 The first step of wild-type retroviral infection is the recognition of a specific cell-surface receptor by the viral envelope, encoded by the env gene.47 The host specificity of retroviruses is therefore primarily dictated by the nature of the envelope proteins that they carry. Each type of retrovirus recognizes a different receptor, but only some of these receptors have been cloned and characterized. For example, murine cells normally express mCAT-1 (a cationic amino acid), which is recognized by the MMLV ecotropic envelope, and human cells express Ram-1 (a phosphate transporter), the receptor for the MMLV 4070 amphotropic envelope. However, not all cell types (not even hematopoietic progenitor cells) are equally well infected with retroviruses. By means of genetic manipulation retroviral vectors can be designed that express different envelope proteins, a method known as "pseudotyping". For example, pseudotyping a retrovirus with the VSV-G envelope allows the entering of
host cells via nonspecific binding to membrane phospholipids and permits the concentration of viral particles by ultracentrifugation. In addition, MMLV vectors can be pseudotyped with truncated HIV glycoproteins to mediate specific gene transfer to CD4+ T cells. Another strategy for altering gene delivery involves decorating the surface of viral particles with agents that display an affinity for cell-surface markers that are different from the natural receptor. A third strategy relies on the genetic modification of the retroviral envelope glycoprotein.

An example of targeted gene transfer is the use of gut-homing T cells as carriers of a therapeutic gene, since these are easily infected with currently available retroviral vectors. We have recently shown that a retroviral vector containing the II-10 gene efficiently transduced human PBMCs and possessed gut-homing properties.

**Future prospects**

GI inflammation is a complex and little understood process. Treatment at present is very often unsatisfactory due to either a lack of efficacy or to side effects. Novel gene-based approaches are being studied but they are still at an early stage of development. Many potential target genes are identified in GI inflammation but we still need safe and efficient methods of delivery to inflamed GI tissues. MMLV based retroviral vectors have been used safely in humans with severe monogenic diseases but their use in GI inflammation has not yet been reported. Experimental data in mice support the idea that inflammation can be treated with T cells retrovirally transduced with a “therapeutic” gene. With regard to T-cells as delivery tools, their survival in vivo and the duration of transgene expression after retroviral infection need to be determined.

With any delivery tool, the efficiency, the ability to target specifically the inflamed tissue, the expression levels of the gene of interest and the safety of the gene-transfer system are important for successful application and acceptance of this strategy in humans. In the last few years, considerable efforts have been made in these areas of research. Retroviral vectors and packaging cells have been modified to improve gene expression and increase viral titres respectively. The study and further use of tissue-
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specific promoters (either constitutive or inducible) and enhancers will allow the design of vectors with a restricted gene expression. Moreover, strategies for modifying gene delivery are under development with a view to improving the infection efficiency of target cells.

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