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Endogenous regulation of a therapeutic transgene restores homeostasis in arthritic joints

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The treatment of chronic inflammatory diseases is complicated by their unpredictable, relapsing clinical course. Here, we describe a new strategy in which an inflammation-regulated therapeutic transgene is introduced into the joints to prevent recurrence of arthritis. To this end, we designed a recombinant adenoviral vector containing a two-component, inflammation-inducible promoter controlling the expression of human IL-10 (hIL-10) cDNA. When tested in vitro, this system had a low-level basal activity and was activated four to five orders of magnitude by various inflammatory stimuli, including TNF-α, IL-1β, IL-6, and LPS. When introduced in joints of rats with recurrent streptococcal cell wall–induced arthritis, the IL-10 transgene was induced in parallel with disease recurrence and effectively prevented the influx of inflammatory cells and the associated swelling of the joints. Levels of inflammation-inducible hIL-10 protein within the joints correlated closely with the severity of recurrence. An endogenously regulated therapeutic transgene can thus establish negative feedback and restore homeostasis in vivo while minimizing host exposure to the recombinant drug.


Introduction

Rheumatoid arthritis is a prototypical chronic inflammatory disease characterized by an imbalance of pro- and anti-inflammatory mediators. Gene therapy has been proposed as a means to restore impaired balance by introducing anti-inflammatory genes into arthritic joints (1). Studies in animal models have shown that gene transfer technology is uniquely suited for therapeutic protein delivery in rheumatoid arthritis (2); two phase I clinical trials in humans are currently underway (3). A major challenge for the anti-inflammatory gene therapy approach remains, however. Since rheumatoid arthritis typically progresses through cycles of relapse and remission, the production of a therapeutic protein must be regulated in order to match the recurrent course of the disease.

A number of vectors with drug-inducible promoters for achieving regulatable transgene expression have been described (4–8). In this approach, transgene expression in vivo is regulated by an orally bioavailable small-molecule drug. However, due to the unpredictable course of the disease, this kind of system will require long-term, largely empirical drug adjustments made by the physician or the patient. Overproduction of anti-inflammatory molecules may be associated with tachyphylaxis, tissue toxicity, or enhanced susceptibility to infection. In addition, experimental evidence indicates that inappropriate timing of anti-inflammatory protein administration can undermine the efficacy of treatment (9). To avoid these problems, we proposed an alternative approach that we call physiologically responsive gene therapy (PRGT) (10). In this approach, anti-inflammatory transgene expression is autonomously regulated by an inflammation-inducible promoter. In this study we assessed the feasibility of using PRGT for treatment of recurrent arthritis. In preliminary studies, we developed a two-component expression system that responds vigorously to inflammatory stimuli in vivo (11). Here, we constructed an adenoviral vector carrying the cDNA of a pleiotropic anti-inflammatory cytokine, human IL-10 (hIL-10), driven by the inflammation-inducible promoter. We demonstrate that intra-articular (IA) delivery of inflammation-regulated hIL-10 effectively prevents recurrence of streptococcal cell wall (SCW) arthritis in rats. The recombinant therapeutic protein is produced in direct relationship to the onset, severity, and duration of inflammation, thus indicating homeostatic regulation of the therapeutic transgene.

Methods

SCW arthritis model. Female Lewis rats were obtained from Charles River Laboratories (Wilmington, Massa-
were stored in 67 mM NaCl, 2.5 mM KCl, 0.5 mM
ments and most of the in vitro studies, Ad viruses
Immediately prior to IA injections, the viruses were
were produced and purified as described (11). After
joint extracts.
nescence of recombinant luciferase in the presence of
samples was controlled by determining chemilumi-
quenchers or enhancers of luciferase activity in the
using a luminometer. The possible influence of
Ad.C3-tat/HIV-luc and Ad.RR5) caused an
increase in joint diameter from day –2 to day 0 but did
not influence the severity of recurrence induced by
PG-APS injection at day 0.

Cell culture. Primary rat synovial fibroblasts were
obtained by enzymatic dispersal of synovial explants
from rats with SCW arthritis (13). Cells were maintained
in RPMI 1640 with 15% FBS and used at passages 5–10.

Adenovirus preparation and storage. Adenoviruses (Ad)
were produced and purified as described (11). After
titration, each virus was frozen and stored at –70°C. Immediately prior to IA injections, the viruses were
thawed and diluted in saline. In two in vivo experiments and most of the in vitro studies, Ad viruses were
stored in 67 mM NaCl, 2.5 mM KCl, 0.5 mM MgCl2, 10 mM Tris (pH 7.4), and 50% glycerol at
–20°C for up to 3 months. Unless otherwise noted, Ad
were injected intra-articularly at day –2, at 106 plaque-
forming units (PFU) per injection. When compared with sham-injected joints, injection of reporter vec-
tors (Ad.C3-tat/HIV-luc and Ad.RR5) caused an
increase in joint diameter from day –2 to day 0 but did
not influence the severity of recurrence induced by
PG-APS injection at day 0.

Cell culture. Primary rat synovial fibroblasts were
obtained by enzymatic dispersal of synovial explants
from rats with SCW arthritis (13). Cells were maintained
in RPMI 1640 with 15% FBS and used at passages 5–10.

Immunodetection methods. Cell lysates were resolved on
da denaturing 15% SDS-polyacrylamide gel and trans-
ferrred to nitrocellulose. hIL-10 was detected with a
cyclonal primary hIL-10 antibody sc-1273 (1:1,000)
(Santa Cruz Biotechnology Inc., Santa Cruz, California,
USA). Immune complexes were visualized using sec-
ondary horseradish peroxidase–conjugated antibody
(1:3,000) and a Renaissance ECL kit (NEN Life Science
Products Inc., Boston, Massachusetts, USA). To assess
hIL-10 in vivo, synovial lavages were pooled (four joints
at each time point), forty microliters of the mixture
were resolved on SDS-polyacrylamide gel and immuno-
detected with hIL-10 antibody, as above. Human
recombinant IL-10 (Biosource International, Camarillo,
California, USA) provided a positive control. hIL-10
ELISA was performed on coded samples using anti-
bodies and recombinant hIL-10 from Pharmingen (San
Diego, California, USA).

Results
The two-component C3-tat/HIV promoter is a broadly respon-
sive sensor of chronic inflammation. In order to achieve
physiological regulation of anti-inflammatory trans-
genes, a promoter element should satisfy certain crite-
ria. First, it should respond to the principal proin-
flammatory mediators. Second, to provide a large
dynamic range of responses, it should have low-level
basal activity and high inducibility. Finally, the fully
activated promoter should be able to drive the produc-
tion of sufficient amounts of recombinant protein to
achieve a therapeutic effect. Transcription of many
acute-phase protein (APP) genes is controlled by
inflammation-inducible promoters, which respond to
inflammatory stimuli in a dose-dependent fashion.
However, the absolute levels of expression achievable by
vectors with endogenous APP gene promoters are dis-
appointingly low compared with levels provided by the
strong constitutive cytomegalovirus (CMV) promoter
(14). In our previous studies, we developed a two-com-
ponent inflammation-inducible expression system.
This chimeric construct uses the promoter of the com-
plement factor 3 (C3) APP gene to control the expres-
sion of the HIV Tat protein; in the same construct, Tat
transactivates the expression of a transgene (rX) linked
to the HIV promoter (11) (Figure 1). Since both the C3
and HIV promoters are cytokine responsive, this bina-
yry construct allows both cis and trans activation of rX
production by inflammatory mediators. When this
binary element was incorporated into a nonreplicating
Ad and injected into mice, it exhibited high inducibili-
ity in several tissues in response to bacterial endotoxin
turpentine oil, and after induction it attained lev-
eels of rX expression comparable to those provided by
the CMV promoter (11).

Here we first examined the ability of the C3-tat/HIV
promoter to respond in vitro to important mediators of
chronic inflammation. Primary synovial fibroblasts
derived from arthritic rat joints were infected with a
reporter Ad (Ad.C3-tat/HIV-luc). Control cells were
infected with the empty vector (Ad.RR5) or with an Ad
that produces luciferase under the control of the CMV
promoter (Ad.CMV-luc). Following adenoviral infec-
tion, serum-starved cells were stimulated with LPS (10 µg/ml), IL-1, IL-6, or TNF-α (each cytokine was used at a concentration of 20 ng/ml). The stimuli activated C3-tat/HIV promoter by approximately four to five orders of magnitude, and the peak levels were comparable to those provided by the CMV promoter (Figure 2, a and b). Of note, CMV-driven luciferase expression was also activated by these stimuli, although the range of induction (approximately twofold) was far less than was seen with the C3-tat/HIV promoter. As IL-1, IL-6, and TNF-α are considered the pivotal cytokines in chronic inflammation, the C3-tat/HIV element thus appears to be a broadly responsive “sensor” of inflammation with a very large dynamic range.

Recurrence of SCW arthritis induces activation of C3-tat/HIV promoter in vivo. To assess the inducibility of the C3-tat/HIV promoter by chronic inflammation in joints in vivo, we first examined the expression of the luciferase reporter during the reactivation phase of SCW arthritis in rats. A distinct feature of this model is the predictability of the recurrence, which reaches a peak at 2 to 4 days and gradually subsides thereafter (12). Ad.C3-tat/HIV-luc and control Ad vectors were injected into primed ankle joints 2 days prior to reactivation of SCW arthritis. In the absence of reactivation, the luciferase activity in the joints injected with Ad.C3-tat/HIV-luc was low, but following reactivation, it achieved the levels attainable with the constitutive CMV promoter (Figure 2c). In three independent experiments, luciferase activity peaked at 2 to 4 days after reactivation; the maximal induction was in the range of 18- to 80-fold compared with unreactivated joints.

Inflammation-inducible production of hIL-10 in vitro. When considering the candidacy of a transgene for a therapeutic protocol, we reasoned that the transgene product should inhibit the numerous inflammatory pathways acting upon the inducible promoter; otherwise, the inducible promoter might remain activated by unchecked proinflammatory mediators. In previous studies we determined that simultaneous suppression of many inflammatory responses, including the induction of IL-1, IL-6, and TNF-α, can be achieved by blocking activation of the transcription factor NF-κB (15, 16). Anti-inflammatory cytokine IL-10 is a potent extracellular antagonist of NF-κB activation (17, 18); in addition, several recent reports have demonstrated the therapeutic efficacy of unregulated IL-10 gene transfer in animal models of rheumatoid arthritis.

Figure 1
The two-component C3-tat/HIV promoter. LTR, long terminal repeat.

Figure 2
The two-component C3-tat/HIV promoter is a broadly responsive sensor of chronic inflammation. (a and b) Induction by the principal proinflammatory cytokines in vitro. Primary synovial fibroblasts were infected in vitro by incubating overnight with the indicated Ad vectors at a multiplicity of infection of 100. Twenty-four hours later, cells were starved in 0.5% FBS medium for 24–48 hours, followed by stimulation for 6 hours with the indicated cytokines (20 ng/ml) or LPS (10 µg/ml). Luciferase activity in cell lysates was normalized to total protein. (a) The levels of inflammation-inducible C3-tat/HIV–driven expression are comparable to those attainable with the CMV promoter. Data are presented as mean of duplicate measurements within an individual experiment; error bars show SD. (b) The C3-tat/HIV promoter provides a large dynamic range of response. The values of fold induction were calculated as the average of readings in independent experiments performed in duplicate (two experiments with IL-1 and IL-6 and three experiments with TNF-α and LPS). (c) In vivo induction following reactivation of SCW arthritis. On day –42, animals were primed by IA injection of PG-APS in both ankle joints. The same joints were injected with the indicated Ad vectors (10⁷ PFU in 10 µl) on day –2. On day 0, arthritis was reactivated by an intravenous injection of PG-APS. Luciferase activity was measured in extracts of pulverized joints (each bar shows the mean ± SD of two joints). In three independent experiments, luciferase activity peaked on day 2–4 and remained elevated through day 4. Unstim., unstimulated; IV, intravenous; rlu, relative luciferase units.
Gene transfer of inflammation-inducible hIL-10 prevents reactivation of SCW arthritis. To assess the therapeutic efficacy of inflammation-inducible hIL-10 expression, PG-APS–primed rat ankle joints were injected with Ad.C3-tat/HIV–hIL-10 or control vectors 1–2 days before arthritis was reactivated by an intravenous injection of PG-APS. The severity of inflammation after reactivation was assessed by measuring joint swelling (13, 16) and the numbers of leukocytes within the joints (24). As determined in two independent experiments, gene transfer of inflammation-inducible hIL-10 afforded efficacious protection against reactivation-inducible leukocytosis (Figure 4a and data not shown). Inflammation-inducible hIL-10 gene transfer also significantly inhibited joint swelling (Figure 4b). Inhibition of joint swelling by Ad.C3-tat/HIV–hIL-10 gene transfer was statistically significant in four independent experiments (Table 1). In one experiment, Ad.C3-tat/HIV–hIL-10 gene transfer did not result in statistically significant suppression of joint swelling (P > 0.05).

Homeostatic regulation of inflammation-inducible hIL-10 expression in vivo. Quantitative assessments of hIL-10 in joint lavages by ELISA showed that, in the absence of reactivation, hIL-10 levels in joints injected with Ad.C3-tat/HIV–hIL-10 were at the lower limit of detection (1 ng/ml). Reactivation of arthritis caused striking increases in hIL-10 levels on days 1 and 2, returning to near-baseline (unreactivated) levels on day 4 (Figure 5a). Since ELISA readings can be influenced by various inhibitors often found in biological fluids, we additionally examined hIL-10 expression using Western blotting. As shown in Figure 5b, qualitative assessments based on Western blotting were in good agreement with the ELISA readings. In contrast to the joints injected with Ad.C3-tat/HIV–hIL-10, hIL-10 production from Ad.CMV–hIL-10 persisted at a constant level throughout the observation period (Figure 5b). The observed sustained expression of Ad.CMV–hIL-10 is consistent with our previous observation that the expression of a CMV-driven reporter green fluorescence protein persisted for at least 1 week after Ad gene transfer into arthritic rat joints (16). This refutes the possibility that the shutoff of inflammation-inducible hIL-10 expression at day 4 was a consequence of an immune response directed against Ad-transduced cells. Another possible explanation is that suppression of the antiapoptotic NF-κB pathway (16) by IL-10 might lead to elimination of Ad-infected cells; however, persistence of CMV-driven hIL-10 expression (Figure 5b) argues against this possibility. Furthermore, inflammation-inducible luciferase expression persisted through day 4 (Figure 2c), strongly suggesting that the rapid decline of Ad.C3-tat/HIV–hIL-10 expression was a direct consequence of hIL-10–mediated inhibition of inflammation, with subsequent downregulation of the inflammation-inducible promoter. We therefore conclude that gene transfer mediated by Ad.C3-tat/HIV–hIL-10 establishes a negative feedback loop similar to those involved in homeostatic regulation of endogenous inflammatory genes.

The severity of recurrent SCW arthritis in rats can be regulated by varying the doses of intra-articularly and intravenously injected PG-APS and the interval between priming and reactivation (12). This allowed us to investigate the relationship between the severity of recurrent arthritis and IA production of inflammation-inducible hIL-10. In three independent experi-
Gene transfer of inflammation-inducible hIL-10 prevents reactivation of SCW arthritis. (a) Inhibition of leukocytosis. On day –42, animals were IA injected with PG-APS in both ankle joints. On day –1, animals were injected in both ankle joints with the indicated Ad, as for Figure 2b. In this experiment there were 12 animals per group on day –1. On day 0, reactivation was induced by intravenous injection of PG-APS. On days 0, 1, 2, 3, 4, and 6 after reactivation, two animals from each group were euthanized, and ankle joints were perfused for determining the numbers of infiltrating white blood cells (WBCs) (21). The time course of leukocytosis is shown. Each bar shows the mean ± SEM of measurements from four joints. Data representative of two experiments are shown. (b) Inhibition of joint swelling. In parallel to measuring leukocytosis, inflammation was assessed by measuring joint swelling. As animals in each group were euthanized in order to obtain joint lavages, the data points represent a diminishing number of joints. Swelling was calculated as (d) – (d0), where (d) and (d0) are mean joint diameters at day -1 and day 0. Error bars represent SEM. P values for comparisons between groups in a and b were calculated using the unpaired one-tailed Student t test. Data on inhibition of joint swelling in other experiments are presented in Table 1.

### Table 1
Therapeutic efficacy of inflammation-inducible hIL-10 gene transfer in SCW arthritis

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ad vector</th>
<th>∆Max. swelling (mm) ± SEM</th>
<th>∆Inhibition of swelling (%) ∆(P value)</th>
<th>∆Max. hIL-10 induction (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1.13 ± 0.25 (n = 10)</td>
<td>59% (P &lt; 0.01)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Ad.C3-tat/HIV-luc</td>
<td>1.28 ± 0.16 (n = 10)</td>
<td>53% (P &lt; 0.05)</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td>Ad.C3-tat/HIV-hIL-10</td>
<td>0.53 ± 0.21 (n = 12)</td>
<td>100% (P &lt; 0.005)</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Ad.C3-tat/HIV-luc</td>
<td>1.61 ± 0.17 (n = 4)</td>
<td>100% (P &lt; 0.005)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Ad.C3-tat/HIV-hIL-10</td>
<td>0.76 ± 0.29 (n = 4)</td>
<td>100% (P &lt; 0.005)</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>Ad.RRS</td>
<td>0.91 ± 0.15 (n = 12)</td>
<td>43% (P &lt; 0.005)</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>Ad.C3-tat/HIV-luc</td>
<td>0.75 ± 0.05 (n = 12)</td>
<td>43% (P &lt; 0.005)</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Ad.C3-tat/HIV-hIL-10</td>
<td>0.63 ± 0.04 (n = 12)</td>
<td>43% (P &lt; 0.005)</td>
<td>70</td>
</tr>
</tbody>
</table>

*Calculated as joint swelling in relation to day 0: ∆d (dmax) = (dmax) – (d0), where (dmax) is the maximal joint diameter throughout the observation period, and (d0) is joint diameter at day 0 (average of n joints). ∆Calculated as ((Δcontrol group) – (Δexperimental group)) X 100%. The significance of difference in reactivation-induced joint swelling between groups was calculated using the unpaired one-tailed Student t test. ∆Mean values of IA hIL-10 were calculated as the average of four joints per group. Maximal values of hIL-10 induction throughout the observation period are shown. ∆Compared with Ad.C3-tat/HIV-luc group. Max., maximal. ND, not determined.
In this context, the novelty and the significance of our work is the demonstration that autoregulatory gene therapy for rheumatoid arthritis is feasible. A distinct advantage of this strategy is that it inhibits inflammation while carrying minimal risk of tissue toxicity or tachyphylaxis from persistent overproduction of the transgene product(s). In this method, compared with other methods for anti-inflammatory drug administration, the risk of unwanted immunosuppression can also be reduced: the drug may be made when, where, in the necessary amount, and for only as long as is necessary for the control of local inflammation.

Our study represents proof of principle rather than a description of a construct immediately available for clinical use. The most serious practical issue remains the selection of a vector for inflammation-inducible gene delivery. Ad vectors are known to induce inflammation within the joint approximately 2 weeks after Ad infection. This limitation precluded examination of the long-term effects of inflammation-inducible expression in the present study. In this regard, use of low-immunogenic vectors such as adeno-associated virus–based vectors (32) may be advantageous, especially because adeno-associated virus–based vector expression is reportedly elevated in the inflamed joints (33–35).

Another concern was that the expression of Tat in our promoter might cause unwanted effects, e.g., cytopotoxicity, immunogenicity, and inflammation. We investigat-ed this in several experiments. In one set of experiments, we compared swelling induced in PG-APS–primed joints by Ad.C3-tat/HIV-luc with that produced by empty Ad.RR5 and Ad.CMV vectors; we found no significant differences (data not shown). In another set of experiments, having compared the severity of PG-APS–induced arthritis recurrence in joints injected with Ad.C3-tat/HIV-luc, Ad.RR5, and saline, we also did not observe significant differences (data not shown). A high-titer (100 PFU/cell) infection of synovial cells in vitro with Ad vectors containing C3-tat/HIV promoter did not cause cytotoxicity (data not shown). As we used low titers in vivo (10^7 PFU/joint, which in our estimation corresponded to less than 1 PFU/cell in vivo), this makes cytotoxic effects of C3-tat/HIV in vivo unlikely.

These observations argue against the cytotoxic and proinflammatory effects of Tat in our experiments. One possible explanation is that many effects of Tat have been attributed to the second exon of tat, which is not present in our construct. Another possibility is that, in the two-component C3-tat/HIV construct, Tat expression is driven by a weak C3 promoter. As we showed previously, even a fully induced C3 promoter provides expression that is two to three orders of magnitude lower than that achieved with strong viral promoters (11, 14); therefore the amount of Tat protein should be much less than that described in most experiments using constitutively overexpressed Tat.

In conclusion, our data indicate that the physiologically regulated transgene supplements an inadequate endogenous anti-inflammatory response, thus becoming an integral part of the negative physiological feedback to inflammation. Potential clinical targets for endogenously regulated gene therapy are not limited to

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**Figure 5**

The expression of inducible hIL-10 transgene in vivo. (a) Reactivated vs. unreactivated joints. Animals with PG-APS–preinjured ankle joints received IA Ad.C3-tat/HIV–hIL-10 on day –2. Arthritis was reactivated in half the animals by intravenous PG-APS (black bars) on day 0. The other half was sham-injected with saline (white bars). The concentrations of hIL-10 in joint perfusates were assessed by ELISA. Each bar shows the mean ± SEM of four joints. In the unreactivated group, hIL-10 concentrations were at or below the limit of detection (1 ng/ml). In a control group (Ad.C3-tat/HIV-luc injected animals), the expression of luciferase has reached a peak at day 4 after reactivation (data not shown). (b) Constitutive vs. inducible transgene expression. In a similar experiment, animals were IA injected at day –2 with the constitutive Ad.CMV-hIL-10 or inducible Ad.C3-tat/HIV–hIL-10. Following reactivation of arthritis (day 0), hIL-10 levels in joint lavages were assessed by Western blotting. Ad.RR5: perfusates of joints injected with an empty Ad. Each lane represents a pool of four joints. In a parallel control experiment, animals were IA injected with Ad.C3-tat/HIV-luc reporter, which expression persisted through day 4 (data represented by Figure 2c). (c) Levels of inflammation-induced hIL-10 correlate with the severity of arthritis. Each point represents data from an independent experiment and is the average of four or more joints (see Table 1); error bars are SEM. Abscissa: maximal SCW-induced swelling in the counterpart control animals (injected with empty or with reporter Ad). Ordinate: maximal average hIL-10 induction [hIL-10 (reactivated joints)/hIL-10 (unreactivated joints)] in Ad.C3-tat/HIV–hIL-10 injected animals.
rheumatoid arthritis; they include a variety of chronic inflammatory diseases, such as Crohn disease and organ transplant rejection (10). It should be possible to regulate the production of many different proteins in this way, using vectors and inflammation-inducible promoters that, like the therapeutic transgene, are tailored to specific clinical settings. The principle of endogenously regulated gene therapy for treating inflammation should therefore not be limited to the promoter system and transgene studied here.

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