Modifications in Adenoviral Coat Fiber Proteins and Transcriptional Regulatory Sequences Enhance Transgene Expression

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ABSTRACT. ***Objective.*** To characterize the adenoviral properties required to enhance intracellular transgene expression for gene therapy.

Methods. Primary human fibroblasts and macrophages were infected with standard replication-defective adenoviruses, adenoviral vectors containing modified fiber coat proteins expressing Arg-Gly-Asp (RGD) or heparin sulfate binding moieties, or a tetracycline-regulatable transgene transcription system. Each of these vectors expressed the ß-galactosidase gene (ß-Gal), which was quantified by flow cytometry. Ankle joints from rats with adjuvant induced arthritis were transduced intraarticularly with each of the vectors and ß-Gal expression was quantified by flow cytometry.

Results. Primary human fibroblasts and macrophages displayed marked increases in transgene expression from both modified fiber protein vectors and from the tetracycline-regulatable vector, compared to an unmodified vector expressing the transgene from the cytomegalovirus promoter/enhancer. In the rat model, the modified fiber protein vectors and the tetracycline-regulatable vector system also displayed increased transgene expression in inflamed rat joints.

Conclusion. Adenovirus attachment and uptake by cells and promoter strength limit transgene expression from conventional adenoviral vectors in models of rheumatoid arthritis. (J Rheumatol 2002;29:1593–600)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
GENE THERAPY
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GENE EXPRESSION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by infiltration of lymphocytes and macrophages into the synovium, hyperplasia of the synovial lining, enhanced angiogenesis, and destruction of cartilage and bone[1]. The normal synovial lining consists of one to 2 cell layers, which increase to 10 or more layers in RA. The cells accountable for the lining increase are macrophages and fibroblasts[2]. Although the agents or factors that regulate synovial hyperplasia have not been fully identified, a number of cytokines present in RA joint fluid or synovial tissue have been shown to recruit monocytes[3], stimulate synovial fibroblast proliferation[4-8], and provide protection against apoptosis[9,10]. Thus to treat RA, gene therapy may be employed to decrease proliferation and/or migration, or to induce apoptosis in the synovial lining macrophage and fibroblast populations.

Gene therapy approaches employing standard cytomegalovirus (CMV) driven replication-defective adenoviral vectors that express soluble factors, including interleukin 10 (IL-10)[11-14], IL-4[15-19], IL-12[20], tumor necrosis factor receptor (sTNFR)[21-23], vascular cellular adhesion molecule-1 (sVCAM-1)[24], and cytotoxic T lymphocyte associated antigen-4 (sCTLA-4)[25], or cell surface molecules such as TNF related apoptosis-inducing ligand (TRAIL)[26] or Fas ligand[27-29], have been shown to suppress experimental inflammatory arthritis. However, few studies have utilized transgenes that express intracellular proteins to prevent the development and progression of arthritis[30-32], and in one study, multiple injections were required to achieve remission of the disease[30]. These data suggest that enhancing infectivity or expression of the adenoviral transgene may allow direct targeting of intra-

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cellular molecules that induce a cytotoxic or cytostatic response in RA, thereby ameliorating the disease.

Recent studies have indicated that replication-defective adenoviruses are poor vehicles for gene transfer in primary human macrophages and fibroblasts[33,34]. Adenoviral vectors may attach to the host cell through the interaction of the adenoviral fiber protein with the coxsackievirus-Ad receptor (CAR) and/or the α2 domain of the major histocompatibility complex class[33,34]. The adenoviral penton base binds to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin receptors through Arg-Gly-Asp (RGD) motifs, which then mediate virus internalization and receptor mediated endocytosis[33,35]. Thus, potential explanations for the decrease in infectivity in these primary cells may be attributed to low levels of CAR, $\alpha_v\beta_3$, or $\alpha_v\beta_5$, suggesting that alterations of the host cell to express increased levels of CAR, $\alpha_v\beta_3$, or $\alpha_v\beta_5$ or modification of the adenoviral vectors to facilitate attachment and infection may result in enhanced expression of the adenoviral transgene. Alternatively, the adenovirus may successfully enter the cell, but the CMV promoter/enhancer may be inactivated or repressed[36-41], decreasing transgene expression. Since the CMV promoter relies solely on endogenous transcription factors to become active, which may limit its activity, employing a binary adenoviral vector system (tetracycline-regulatable system) may be a more efficient approach to yield enhanced transgene expression.

We found that modifying the adenoviral vector fiber protein or employing a regulatable (tetracycline) promoter significantly enhanced the number of infected cells and the intensity of the transgene in primary host cells *in vitro* and in a rat model of inflammatory arthritis. All adenoviral vectors employed in the study were standard Ad5 replication-defective vectors. The control adenoviral vector expressing a normal fiber protein (CMV-ß-Gal) was compared with modified fiber protein vectors expressing RGD (CMV-ß-Gal-RGD) or heparin sulfate binding moieties[33] (CMV-ß-Gal-PolyL), which allow for increased attachment to the cell. Additionally, a tetracycline-regulatable system was employed, which utilizes a mutant form of the *Escherichia coli* tetracycline repressor fused to the C-terminal domain of the eukaryotic transcriptional activator VP-16 (rtTA). This factor transactivates a second adenoviral vector that expresses ß-galactosidase (ß-Gal), under the control of a minimal promoter containing multiple tetracycline operon sites, only in the presence of tetracycline or its analogs[42-44] (Tet-ß-Gal/CMV-rtTA). Normal human dermal fibroblasts and monocyte-differentiated macrophages displayed a marked increase in the number of ß-Gal positive cells and the intensity of the ß-Gal transgene when transduced with Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), or Ad-Tet-ß-Gal/Ad-CMV-rtTA compared to control Ad-CMV-ß-Gal. Further, in the rat model of inflammatory arthritis Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), or Ad-Tet-ß-Gal/Ad-CMV-rtTA induced a 154 ± 38%, 61 ± 24%, or 72 ± 34% increase in ß-Gal expression, respectively, compared to Ad-CMV-ß-Gal. Thus these data show that either modification of the fiber protein to enhance viral attachment to host cells or a regulatable promoter system resulted in superior transgene expression compared to standard CMV-driven adenoviral vectors.

MATERIALS AND METHODS

Cell isolation and culture. Buffy coats (Lifesource, Glenview, IL, USA) were obtained from healthy donors. Mononuclear cells, isolated by Histopaque (Sigma, St. Louis, MO, USA) gradient centrifugation, were separated by countercurrent centrifugal elutriation (JE-6B, Beckman Coulter, Palo Alto, CA, USA) in the presence of 10 μg/ml polymyxin B. Isolated monocytes were ± 90% pure as determined by morphology, nonspecific esterase staining, and CD-14 (Becton Dickinson, Franklin Lakes, NJ, USA) expression examined by flow cytometry (data not shown). Monocytes were further enriched by adherence and differentiated *in vitro* for 7 days in RPMI containing 20% heat inactivated fetal bovine serum (FBS), 1 μg/ml polymyxin B sulfate, 0.35 mg/ml L-glutamine, 120 U/ml penicillin and streptomycin (20% FBS/RPMI)[45-47]. Seven-day differentiated macrophages strongly expressed maturation markers including CD71 and the integrin $\alpha_v\beta_5$, which is necessary for adenoviral infection of macrophages[48]. Normal human dermal fibroblasts were purchased from American Type Culture Collection (CRL 1475, ATCC, Rockville, MD, USA). Fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Life Technologies, Gaithersburg, MD, USA). When indicated, doxycycline hydrochloride was added to infected cells (200 ng/ml) or single dose was inject intraperitoneally (2 mg/500 g).

Recombinant adenoviral vectors expressing ß-galactosidase. Ad-CMV-ß-Gal[49,50], Ad-CMV-ß-Gal (PolyL) (GenVec Inc., Rockville, MD, USA)[33], and Ad-CMV-ß-Gal (RGD) (GenVec)[33] are first generation adenoviral vectors deleted for the E1 and E3 adenoviral genes and encode ß-Gal under the control of the CMV promoter. Ad-CMV-ß-Gal (PolyL) and Ad-CMV-ß-Gal (RGD) express a mutated fiber gene that adds 7 lysine moieties or an RGD integrin-binding sequence, respectively, to the carboxyl terminus of the fiber protein[33]. Tetracycline-inducible gene expression was achieved with a binary adenovirus strategy as described[44]. Both vectors, Ad-Tet-ß-Gal and Ad-CMV-rtTA, are also first-generation adenoviral vectors deleted for the E1 and E3 adenoviral genes. Ad-Tet-ß-Gal encodes ß-Gal under the control of a minimally active CMV promoter downstream of 7 tetracycline operator sites. Ad-CMV-rtTA expresses a chimeric transcription factor composed of the mutant tetracycline repressor fused to VP16-transactivation domain from the CMV promoter/enhancer. The viral preparations used for both *in vitro* and *in vivo* studies were purified by CsCl gradient centrifugations, dialyzed against buffer containing 10 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, and 135 mM NaCl and stored at –80°C in 10% glycerol. Since the concentration of the modified fiber protein vectors cannot be determined by plaque assay because they yield small plaques that cannot be measured accurately[33,51,52], adenoviral concentration was determined by measuring optical density at 260 nm[53,54]. The viral particle concentration was determined using the extinction coefficient 1 OD = 10^{12} viral particles (vp)/ml[54]. Thus the vp count was used to determine the concentration of virus added in each experiment. Plaque assays were also performed on the vectors that did not possess the modified fiber proteins. The concentrations of these vectors by plaque assay and by vp count corresponded very closely: Ad-CMV-ß-Gal, 1.2×10^{11} plaque forming units (pfu)/ml (vp/ml by OD = 1.75×10^{13}); Ad-CMV-rtTA 1.05×10^{11} pfu (vp/ml by OD = 1.28×10^{13}); and Ad-Tet-ß-Gal 1.09×10^{11} pfu (vp/ml by OD = 9.9×10^{12}). These observations indicate that our method of isolation resulted in viral particles that were comparably infectious.

Adenovirus infection. Seven-day differentiated macrophages were infected with 10, 50, 100, 200 vp/cell for 2 h in serum-free RPMI. Following infection, 20% FBS/RPMI was added at a 1:1 ratio (up to 10% FBS) for an additional 12 h. The infected macrophages were then washed gently with PBS and cultured in 20% FBS/RPMI for an additional 24 h. For fibroblast infection, cells were plated in growth medium (10% FBS) and allowed to attach before

being transferred to low serum medium. Cultures were serum starved in 0.5%FBS/DMEM for 2 days prior to infection. Fibroblasts were then counted and cultures were infected with 25, 50, 100, 200 vp/cell for 12 hours in low serum medium. At the end of the infection period the virus was removed by washing with PBS and returned to low serum medium for an additional 12 h. The cultures were then stimulated for an additional 24 h by the addition of medium containing 10% FBS. ß-Gal positivity and intensity (mean fluorescence intensity) were determined by flow cytometry as described[55]. Briefly, cells were washed in PBS and suspended in loading buffer (PBS, pH 7.4, 4% FCS, 10 mM Hepes) for 10 min at 37°C. An equal volume of loading buffer plus 5 mM of fluorescein digalactoside (FDG, Molecular Probes, Eugene, OR, USA) was added to cells for 1 min at 37°C. Five volumes of loading buffer were added to the cells prior to analysis by flow cytometry. The percentage of ß-Gal positivity and intensity was determined by flow cytometry using a Beckman-Coulter Epics XL flow cytometer and System 2 software (Robert H. Lurie Cancer Center, Northwestern University). Objects with minimal light scatter were excluded since they may represent debris, and nonviable cells were identified by the inclusion of propidium iodide. No differences in viability were observed between samples infected with the different vectors.

Adenoviral delivery to normal knees. Normal knees were infected with a single dose of 1×10^9 viral particles/20 μl, injected anteriorly into the knee joint space. Three days after infection, rats were euthanized and the synovium was isolated, frozen in OCT, and 5 μm sections were analyzed by immunohistochemistry to determine ß-Gal expression. Briefly, sections were fixed in 4% paraformaldehyde, washed 3 times with ice-cold PBS, and stained with 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-Gal, Sigma) containing solution [10 mM sodium phosphate (pH 7.0), 1 mM $MgCl_2$, 150 mM NaCl, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, and 0.1% Triton X-100] in a humid chamber at 37°C until a blue color appeared as observed under a microscope with bright-field illumination. Sections were examined and photographed on a Nikon ES400 microscope.

Adjuvant induced arthritis. Female Lewis rats, (100–150 g) were injected intradermally at the base of the tail with 0.3 ml of *Mycobacterium butyricum* in mineral oil solution (10 mg/2 ml mineral; adjuvant). At 21 days after adjuvant injection a single dose of adenovirus (1×10^9 viral particles/20 μl) was injected anteriorly into the ankle joint space as described[19]. In a parallel study, at this time point, all ankles displayed physical and histological features of arthritis[56]. An increase in inflammation following adenoviral delivery to ankles or knees was not observed, similar to previous studies[30,31,57]. Three days after adenoviral infection, rats were euthanized and ankles were amputated. The synovial membranes were exposed to digestion buffer containing collagenase (250 units/ml), dispase (2.4 mg/ml), and DNAse I (10,000 dornase units/ml) in Hanks' buffered solution (pH 7.2–7.4) at 37°C for 2–3 h. After washing with PBS and filtering the cells through a mesh strainer, single cell suspensions were isolated[58-60]. Cells were then analyzed for ß-Gal expression and activity by flow cytometric analysis employing the FDG substrate. Nonviable cells were excluded by the incorporation of propidium iodide. No differences in cell viability were observed between the isolated cell suspensions.

Statistical analysis. Results were expressed as the mean ± standard error. Differences between groups were analyzed by unpaired 2 tailed Student's t test.

RESULTS

Fibroblasts and macrophages make up the synovial lining and are vital contributors to the pathogenesis of RA. Investigations have shown that fibroblasts and macrophages are also poorly infected by standard Ad5 vectors[33,34]. Therefore, we compared the number of ß-Gal positive cells and ß-Gal intensity following transduction with adenoviral vectors that have modifications of the fiber protein, or express ß-Gal under a tetracycline-inducible promoter to the standard Ad5, CMV driven vector. Primary human dermal fibroblasts were infected with Ad-CMV-ß-Gal, Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), or Ad-Tet-ß-Gal/Ad-CMV-rtTA at various concentrations. The percentage of ß-Gal positive cells and the intensity of ß-Gal were determined by flow cytometric analysis employing the FDG substrate. At 10 vp/cell, 12 ± 0.8% of the CMV-ß-Gal infected cells were positive, while 88 ± 6.0% of the CMV-ß-Gal (PolyL), 36 ± 6.0% of the CMV-ß-Gal (RGD), and 18 ± 2.0% of the Tet-ß-Gal/CMV-rtTA were positive (Figure 1A). However, at 50 vp/cell, 99% of the CMV-ß-Gal (PolyL), 93 ± 0.8% of the CMV-ß-Gal (RGD), and 97 ± 3.0% of the Tet-ß-Gal/CMV-rtTA were positive for ß-Gal (Figure 1A). In contrast, the control CMV-ß-Gal infected cells exhibited only 22 ± 0.5% positive ß-Gal positivity, which peaked at 200 vp/cell (60 ± 2.0% ß-Gal positivity). In addition, CMV-ß-Gal (PolyL), CMV-ß-Gal (RGD), and Tet-ß-Gal/CMV-rtTA displayed a marked increase in the intensity of ß-Gal compared to CMV-ß-Gal (Figure 1B). An 18-fold, 23-fold, and 26-fold increase in intensity of ß-Gal expression by cells infected with CMV-ß-Gal (PolyL), CMV-ß-Gal (RGD), and Tet-ß-Gal/CMV-rtTA, respectively, was observed at 200 vp/cell compared to cells infected with the control adenovirus. A similar pattern of enhanced intensity was also detected in normal human monocyte derived macrophages (Figure 1C). Thus, these data suggest that adenoviral vectors expressing modified proteins or a regulatable promoter resulted in enhanced transgene expression in primary human fibroblasts and macrophages.

Normal rat knees were employed to examine the *in vivo* expression of the transgene from the modified vectors. Normal rat knee joints were injected with each of the vectors. Three days after infection, rats were euthanized and the knees were amputated. The synovium was dissected out, frozen, and analyzed for ß-Gal staining. Histological examination revealed enhanced expression of ß-Gal in Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), or Ad-Tet-ß-Gal/Ad-CMV-rtTA, compared to Ad-CMV-ß-Gal transduced joints (Figure 2).

For a more quantitative assessment of transgene expression and to determine if differences could be identified in an inflamed joint, we examined ankles of rats with adjuvant induced arthritis. Twenty-one days after the induction of adjuvant induced arthritis, when all ankles displayed physical and histological features of arthritis[56], rat ankles were injected with each vector. Three days after infection, rats were euthanized and ankles were amputated, the synovium exposed to digestion solution, and the single cell suspensions were analyzed for ß-Gal expression by FACS. The number of ß-Gal positive cells was significantly increased ($p < 0.0.04$ to 0.02) with each of the modified vectors (Figure 3A). The Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-Gal/Ad-CMV-rtTA displayed 154 ± 38%, 61 ± 24%, and 72 ± 34% increases in intracellular ß-Gal expression, respectively, compared to Ad-CMV-ß-Gal (Figure 3A). Further, the intensity of

ß-Gal staining in the Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-Gal/Ad-CMV-rtTA was markedly enhanced compared to Ad-CMV-ß-Gal (Figure 3B). Thus, these data corroborate the *in vitro* data, indicating that modifications of the fiber protein or employing a regulatable promoter enhanced expression and intensity of transgene expression.

DISCUSSION

The vast majority of studies that have examined the limited transgene expression *in vivo* by replication-defective adenoviral vectors have focused on their role in inducing an immune response following infection[61,62]. We demonstrated that in primary human fibroblasts and macrophages, adenoviral infection with vectors modified to express RGD motifs or heparin sulfate binding motifs displayed a significant and dramatic increase in the number of infected cells and the intensity of the expressed transgene compared to standard Ad5 vector. Although over 90% of the Ad-CMV-ß-Gal (PolyL) and Ad-CMV-ß-Gal (RGD) infected cells were ß-Gal positive at 10 and 50 vp/cell, increasing the viral concentration resulted in enhanced intensity of the transgene expression. However, we cannot exclude the possibility that the differences observed may be due to increased expression, if the number of viral particles entering the cells did not change. These data indicate that increasing the number of modified viral particles per cell increased the intensity of transgene expression, even though all cells were infected at the lower concentrations of virus.

Employing the binary adenoviral vector system, where one vector regulates another in the presence of an activator (doxycycline), also resulted in increased transgene expression compared to the standard CMV driven adenoviral vector. This suggests that the increased frequency of cells expressing ß-Gal, both *in vitro* and *in vivo*, was due to enhanced expression of the transgene by the regulatable promoter system. Thus a regulatable promoter system may overcome weak or repressed expression compared to the CMV promoter alone. Further, employing the Tet-expression system, transgene expression was highly regulated, since in the absence of tetracycline or the rt-TA vector, minimal or nondetectable levels of ß-Gal were observed (data not shown), similar to previous investigations[42-44]. However, the substantial enhancement of transgene expression by the modified fiber protein expressing adenoviral vectors indicates that the low activity of the CMV promoter in the examined cells may be mitigated by the increased adherence and infectivity of the modified adenoviral vectors.

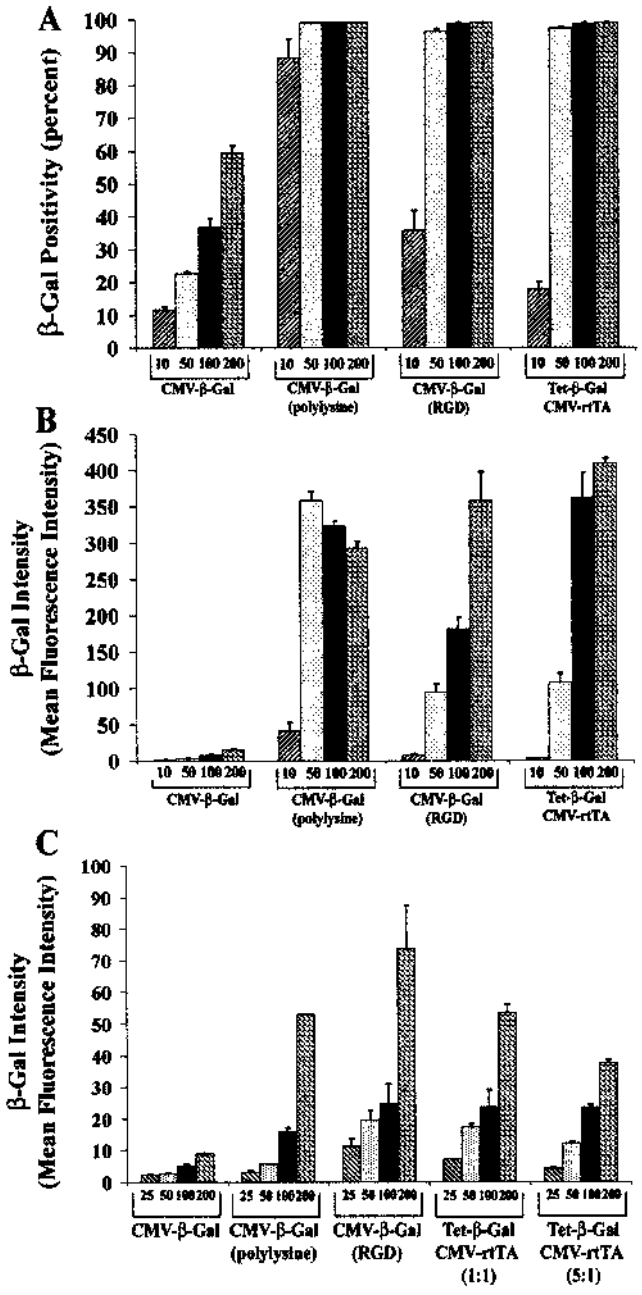

Figure 1. Comparison of Ad-CMV-ß-Gal, Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-gal/Ad-CMV-rtTA in fibroblasts and macrophages. A, B. Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-gal/Ad-CMV-rtTA infected cells display a marked increase in the number of ß-Gal positive cells and the intensity of ß-gal compared to Ad-CMV-ß-Gal. Triplicate cultures of human dermal fibroblasts were made quiescent for 48 h in 0.5% FBS/DMEM. Cells were transduced with the indicated adenovirus with 10, 50, 100, or 200 viral particles/cell for 12 h, then the virus was removed and cultures were returned to 0.5% FBS/DMEM for an additional 12 h. 10% FBS/DMEM plus doxycycline hydrochloride (DOX) (200 ng/ml) was then added for 24 h and cells were analyzed by flow cytometry to detect ß-Gal expression using the FDG substrate. Values represent the mean ± standard error from a representative of 7 independent experiments. C. Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-gal/Ad-CMV-rtTA infected primary macrophages exhibit enhanced ß-Gal expression compared to Ad-CMV-ß-Gal. Seven-day differentiated macrophages were infected for 2 h in serum-free RPMI. After infection, 20% FBS/RPMI was added at a 1:1 ratio (up to 10% FBS) for an additional 12 h. The infected macrophages were then washed gently with PBS and cultured in 20% FBS/RPMI plus DOX (200 ng/ml) for an additional 24 h. Cells were analyzed by flow cytometry to detect ß-Gal expression using the FDG substrate. Values represent the mean ± standard error from a representative of 4 independent experiments.

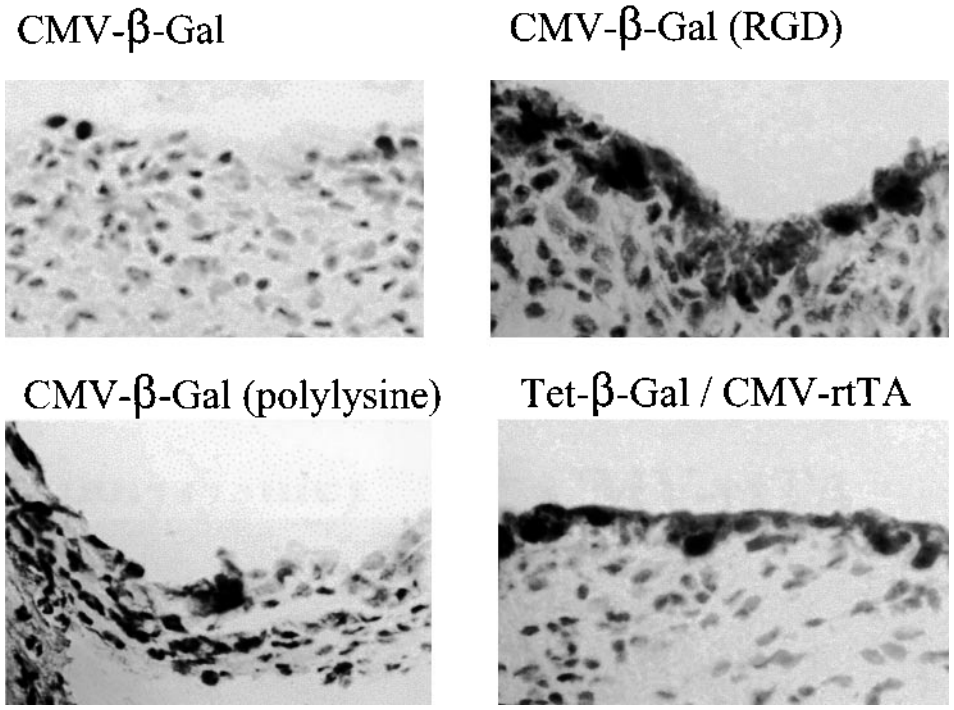

Figure 2. Increased ß-Gal expression in Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-gal/Ad-CMV-rtTA infected rat synovium compared to Ad-CMV-ß-Gal. A representative photomicrograph shows infected rat synovium stained with X-Gal (blue) and counterstained with hematoxylin (purple). Untreated female Lewis rat knees (4 knees/adenovirus) were injected intraarticularly with the indicated adenovirus (1×10^9 viral particles). For the Ad-Tet-ß-Gal/CMV-rtTA, a single dose of DOX (2 mg/500 g) was injected intraperitoneally after adenoviral infection. Three days postinfection, rats were sacrificed, the synovium was isolated from knees, frozen, and stained with X-Gal.

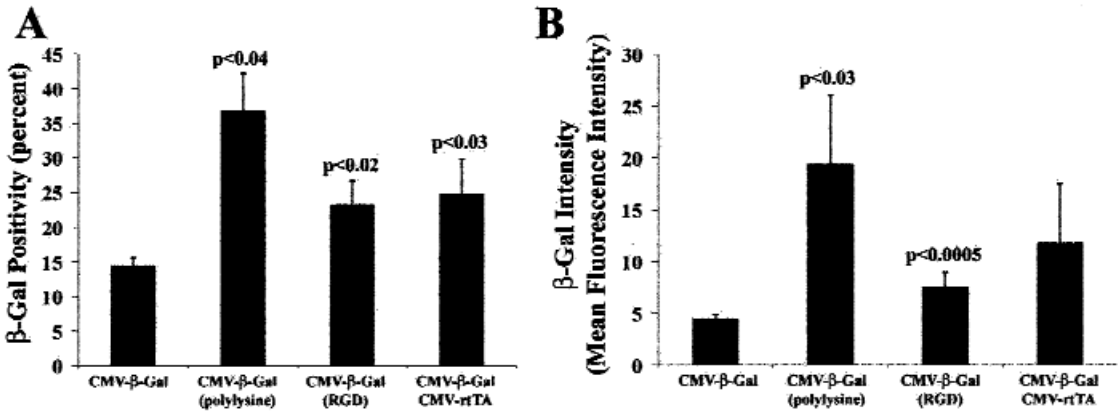

Figure 3. Ad-CMV-ß-Gal (PolyL), Ad-CMV-ß-Gal (RGD), and Ad-Tet-ß-Gal/Ad-CMV-rtTA infected rat AIA ankles display increased number of ß-Gal positive cells compared to Ad-CMV-ß-Gal. Twenty-one days after adjuvant injection, female Lewis rat ankles (14 ankles/adenovirus) were injected intraarticularly with the indicated adenovirus (1×10^9 viral particles). For the Ad-Tet-ß-Gal/CMV-rtTA, a single dose of DOX (2 mg/500 g) was injected intraperitoneally after adenoviral infection. Three days postinfection, rats were sacrificed, and the ankle synovium was exposed to digestion buffer and examined by flow cytometry to detect ß-Gal infectivity (A) or intensity (B) using the FDG substrate. Values represent the mean ± standard error of 14 ankles and were compared for statistical significance by unpaired 2 tailed Student t test. P values are relative to CMV-ß-Gal treated ankles.

Collectively, these data suggest that transgene expression may be enhanced by modulating viral entry or promoter activity.

Multiple transcription factors may activate or repress the CMV promoter, including nuclear factor-κB (NF-κB), CREB/ATF, NF-1, AP-1, SP1, MDBP, and YY1[63]. One of the more vital transcription factors regulating the CMV promoter is NF-κB. Although NF-κB is constitutively activated in primary vascular smooth muscle cells (VSMC), the induction of NF-κB activity by forskolin or phorbol esters enhanced adenoviral transgene expression[36], suggesting that the basal level of NF-κB may not be sufficient to induce the CMV promoter or that the promoter may be repressed in VSMC following infection. Further, adenoviral infection of mouse livers revealed undetectable expression of the transgene 2 weeks after infection, suggesting that the CMV promoter may be inactivated. However, the addition of lipopolysaccharide, which activates NF-κB, reactivated the promoter, and transgene expression was readily detectable[63]. In contrast, a hybrid promoter consisting of a minimal CMV promoter and the enhancer element from hepatitis B virus was active for at least 11 weeks in mouse livers[63], suggesting that the CMV promoter may be repressed in hepatocytes *in vivo*. Similarly, the CMV promoter may also be partially repressed in primary human fibroblasts and macrophages. We observed that a macrophage-specific promoter was more effective in macrophages compared to the CMV promoter[64], further supporting a role for cell type-specific promoters or regulatable promoters for future gene therapy studies. Although transgene expression was detectable in cells infected with Ad-CMV-ß-Gal, Ad-Tet-ß-Gal/Ad-CMV-rtTA transduced cells exhibited a marked increase in the intensity of the transgene expression, suggesting that the Tet-regulatable system may be more efficient for regulating transgene expression. Thus, compared to a vector driven by the regulatable promoter, the reduced transgene expression observed with the standard CMV-driven vector may be attributed to inactivation or partial suppression of the CMV promoter, since both vectors are standard Ad5 vectors and the mode of infection is identical.

Although additional investigations have shown that modification of the protein fiber increases transgene expression *in vivo*[33,51,65-67], to date no study has directly quantified on a cellular basis the percentage expression and the intensity of the transgene *in vivo*. Further, no study has directly investigated the effectiveness of binary adenoviral vectors compared to adenoviral vectors with a modified protein coat. Our results corroborate previous investigations[33,34], since enhanced infection of fibroblasts and macrophages was observed with modified fiber protein-expressing adenoviruses. However, employing a regulatable promoter also markedly enhanced expression of the transgene compared to control Ad5 vector, suggesting that modification of the fiber protein alone is not sufficient to provide optimal transgene expression. Although our studies utilized a reporter gene to determine expression levels, future investigations may employ intracellular molecules or cell surface ligands that are known to suppress arthritis, such as IκBα[68-70] or Fas ligand[27-29], in the vectors with regulatable promoters or modified fiber protein coats. Thus, an adenoviral vector with a modified protein fiber and a transgene that is under the control of a regulatable promoter may be optimal for gene therapy that targets an intracellular signaling pathway, particularly in a localized area such as the RA joint.

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