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Enhancement of Adenovirus-Mediated Gene Delivery to Rheumatoid Arthritis Synoviocytes and Synovium by Fiber Modifications: Role of Arginine-Glycine-Aspartic Acid (RGD)- and Non-RGD-Binding Integrins1

Myew-Ling Toh,* Saw-See Hong,† Fons van de Loo,§ Laure Franqueville,† Leif Lindholm,‡ Wim van den Berg,§ Pierre Boulanger,2,2 and Pierre Miossec2,*

Rheumatoid arthritis (RA)1 is characterized by chronic synovial inflammation and pannus formation leading to joint destruction. Biological agents such as anti-TNF therapies are efficacious, but may be limited by cost and systemic side effects. Gene therapy has been developed as an alternative approach in targeted delivery of therapeutic agents. Among several viral vectors used, nonreplicative adenovirus (Ad) serotype 5 (Ad5) vectors are relatively safe and well studied in animal models. Although the efficacy of gene therapy in animal models of arthritis has been demonstrated, applicability to human disease is less well documented (1–3). Key issues include efficient gene delivery, reducing vector dose and vector-induced inflammation and toxicity. The Journal of Immunology, 2005, 175: 7687–7698.

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described. Defining these mechanisms may enable the development of improved vectors to specific synovial targets.

In diseases such as cancer and cystic fibrosis, also hampered by the absence of CAR in cells and tissue, efforts to improve vector efficiency have focused on genetically modifying the knob domain of Ad5 to achieve CAR-independent cell entry (19–24). In arthritis, this has been less well explored. Approaches have included retargeting of Ad5 vectors to alternative receptors by insertion of the integrin-binding RGD motif in the HI loop or at the C terminus of the fiber, or by replacement of the Ad5 fiber by the fiber of another serotype (9, 13, 25). Recently, an RGD-ligated Ad5 increased efficiency of transduction of the IL-1 receptor antagonist with improved anti-inflammatory effects in murine collagen-induced arthritis (CIA) (13, 26).

An alternative strategy to improve Ad5 vector tropism in CAR-negative cells is to shorten the fiber shaft (8, 9, 19, 27). Members of subgroup B Ad such as Ad3, which have naturally short fibers (e.g., serotypes 11, 16, 35), or chimeric Ad5 vectors carrying subgroup B fibers more efficiently transduced RA FLS compared with Ad5 (9, 14). In the present study, we describe the use of short fiber-modified Ad5 vectors in efficient gene transfer to CAR-negative human RA FLS in vitro, synovium ex vivo explants, and murine CIA in vivo. We also defined the receptor usage and mechanism of viral entry in RA FLS.

Materials and Methods

Cells, synovium explants, and synovial fluid (SF)

FLS were obtained from synovial tissue from RA patients undergoing joint surgery who fulfilled the American College of Rheumatology criteria for RA (28). FLS were isolated by enzyme digestion and cultured as described elsewhere (29, 30). Cells beyond the third passage were >99% negative for CD11c, CD3, CD19, CD14, and HLA-DR, and positive for CD10, CD44, and CD54 (BD Biosciences), and were used between passages 4 and 9. An ex vivo synovium explant model was prepared as described (31, 32). In brief, fat and fibrous tissue were removed from freshly harvested synovial tissue and cut into small explants with a volume of ~3 mm³. Explants were cultured in quadruplicate in 6-well plates in DMEM/10% FCS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine, at 37°C in a humidified 5% CO₂ incubator. After 3–4 days, a cellular outgrowth characterized as FLS are observed migrating out from the edge of synovial explants. FLS were characterized as described above. All data are the results of at least three separate experiments using FLS and synovial tissue from three different human RA donors. A549 cells (lung alveolar carcinoma; CCL 185) were provided by the Clinical Virology Laboratory (Hospital de de Lyon, Domaine Rockfeller, Lyon, France). SF samples from six women with RA (mean age, 45 years old; 70% rheumatoid factor positive) were collected and stored at −20°C.

Recombinant adenoviral vectors

Ad5 vectors were EI-deleted, with the E1 region replaced by a GFP expression cassette under the control of a CMV promoter. A conventional Ad5 vector, Ad5GFP-FiWT carrying wild-type (WT) fibers with 22 shaft repeats, was used as a control vector and propagated in the E1-transcomplementing cell line HEK-293 (abbreviated 293 cells). Short fiber-modified vectors were propagated in the dual E1+ fiber-trans-complementing cell line (referred to as 293-Fiber cells; Ref. 22), then in 293 cells for the last amplification step (19). The construction of short fiber-modified vectors has been described elsewhere (19, 22, 33).

In brief, the Ad5 fiber backbone contained the following domains from N terminus to C terminus: 1) the fiber tail; 2) seven shaft repeats; 3) a nonviral transduction signal (PVDAVSLRQVQAELGQVQHQLQAFS YYKVKELPFPNG) called the neck region peptide from the human lung surfactant protein D; 4) a tridecapeptide linker sequence (AKKLNDA QAPKSD); and 5) various C-terminal sequences. Ad5GFP-R7-knob carried the native knob domain at the C terminus of the shortened shaft (Fig. 1A), whereas “knobless” vectors Ad5GFP-R7-RGD carried a linear RGD peptide (Fig. 1C), and Ad5GFPΔknob carried no ligand (Fig. 1D). Virus stocks were purified by CsCl ultracentrifugation (34). Infectious titers (concentrations of infectious virions) were determined by plaque titration assay on CAR and integrin-expressing 293 cells, and values were expressed as PFU per milliliter. The number of physical virus particles (physical virus particles = noninfectious + infectious virions) in virus stocks was determined using a Bradford protein assay (Bio-Rad) with BSA (Bio-Rad) as the standard, taking into account the mass for a single virion (2.9 × 10⁻⁶ g) and the value of 3.4 × 10¹⁵ virions/mg total protein (22). Data was expressed as physical virus particles per milliliter.

In vitro transduction of RA FLS and ex vivo transduction of synovium explants

Cells and tissues were infected with Ad5GFP-FiWT or fiber-modified vectors Ad5GFP-R7-knob, Ad5GFP-R7-RGD and Ad5GFPΔknob at a multiplicity of infection (MOI) of 2, 20, or 200 PFU/cell for 24–72 h, and GFP expression measured by flow cytometry (fluorescence activator cell sorter scan; BD Biosciences) or fluorescence microscopy using an Axiovert 135 microscope (Zeiss) equipped with an AxioCam camera. Briefly, RA FLS were washed with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) then analyzed directly by fluorescent microscope. For flow cytometry analysis, RA FLS were washed in PBS, detached using trypsin-EDTA, fixed in 4% paraformaldehyde, and analyzed for GFP expression. Gene transduction efficiency was measured in the presence and absence of TNF-α (10 ng/ml) or IL-1β (10 ng/ml) (Sigma-Aldrich).

Identification of Ad5 cell surface receptors and receptor usage in RA FLS

Construction and isolation of recombinant Ad5 proteins hexon, knob, WT Pβ, and its two substitution mutants R340E and D288K have been described in detail elsewhere (35–37). R340E carries an Arg-to-Glu substitution at position 340 in the RGD motif, and D288K an Asp-to-Lys substitution at position 288 in the LDLV motif. Receptor usage was determined by cell binding competition assays using viral proteins as competitors (WT

FIGURE 1. Schematic representation of adenoviral vectors carrying the WT fiber or recombinant short fibers. The fiber tail domain is shown by □, the shaft domain by □, the extrinsic trimerization motif by □, and the knob domain by ○. A, Control Ad5 vector (Ad5GFP-FiWT) carried WT fibers that consisted of the following domains from the N terminus to C terminus: a tail domain linked to the Pβ, followed by 22 shaft repeats, and an intrinsic trimerization domain including the knob domain. B–D, Ad5 vectors with short- shafted fibers consisted of the tail domain, the shortened shaft with seven repeats, followed by an extrinsic trimerization motif. The fiber shaft was terminated by the native knob domain as in Ad5GFP-R7-knob (B), a linear RGD sequence as in Ad5GFP-R7-RGD (C), or an opal stop codon as in Ad5GFPΔknob (D).
Pb, R340E and D288K mutant, hexon or fiber knob), as described previously (22, 23). Briefly, 1 x 10⁴ RA FLS were incubated with hexon (3, 30, or 300 ng total protein), Pb protein, WT or mutant (0.5, 5, or 50 ng), or knob protein (4, 40 or 400 ng) for 1 h at 4°C, in 200 μl/well of a 24-well plate. These amounts were estimated to be equivalent to 10⁴-, 10⁻²-, and 10⁻⁰-fold excess over the hexon capsid proteins present in the infectious viral inoculum, 3-, 30-, and 300-fold excess over the Pb capsomers, and 10⁻⁴-, 10⁻¹-, and 10⁻⁰-fold over the knob content, respectively. This was based on the prevalence of 40 copies of Pb, and 12 copies of fiber knob per virion. Cells were washed in cold PBS then incubated with AdsGFP-R7-knob at a MOI of 200 for a further 30 min at 4°C. Cells were washed and transferred to 37°C overnight, and GFP expression was measured by flow cytometry.

Rabbit polyclonal anti-Pb Ab was used to inhibit viral Pb-integrin recognition in gene transfer assays (35, 36). AdsGFP-R7-knob at a MOI of 200 was preincubated with anti-Pb Ab or control preimmune rabbit serum at 3 and 30 μg/ml for 1 h at 4°C, then complexes were added to FLS for 30 min at 4°C. Cells were washed in PBS and transferred to 37°C overnight, and GFP expression was measured by flow cytometry.

Integrin expression was determined by flow cytometry using specific mAbs against α₁β₁ (CD51/CD61; BD Pharmingen), α₁β₅ (CD49E; Chemicon International), α₅(1C2), α₅(1B5; Chemicon International), and β₃ integrins (JSB5; Chemicon International). RA FLS were incubated with 40 μg/ml mAbs against integrins for 30 min at 4°C, washed with PBS, and incubated with FITC-conjugated goat anti-mouse Ab (BD Pharmingen) for 30 min at 4°C. Control samples were incubated with secondary Ab conjugate alone. For function-blocking integrin assays, RA FLS were incubated with 40 μg/ml mAbs against integrins, α₅β₅ (CD69), α₅β₅ (P3G2), anti-α₅ (1C2), anti-α₁ (1B5), anti-β₃ (1B5; Chemicon International) for 1 h at 4°C, and cells were washed in PBS then incubated with AdsGFP-R7-knob at a MOI of 200 for 30 min at 4°C (38). Cells were washed and returned to 37°C overnight, and GFP expression was measured by flow cytometry.

**Virus neutralization by SF**

SF from six RA patients was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and incubated for 30 min at 35°C to inactivate virus. To determine the neutralizing activity, SF was preincubated with mAbs against integrins, 1/10, 1/100, and 1/1,000, AdsGFP-FWt, AdsGFP-R7-knob, and AdsGFPΔknob were incubated with SF dilutions for 1 h, then added to RA FLS for 1 h at 37°C. Supernatants were removed, RA FLS were incubated with fresh medium, and GFP expression was measured by flow cytometry after 24 h. IgG was depleted from SF as described previously (39). Briefly, SF was incubated overnight at 4°C with protein G-Sepharose affinity gel (Amersham Biosciences), IgG-protein G-Sepharose complex was removed by centrifugation at 12,000 rpm for 5 min, then 10-fold dilutions of IgG-depleted SF were incubated with AdsGFP-FWT and added to RA FLS as described above.

**CIA model**

CIA was induced in male (8–10 wk old) DBA/1 mice (Bomholtgard Breeding and Research Center) with bovine type II collagen (bCII) as described previously (40). All in vivo studies complied with national legislation and were approved by local authorities of the Care of Use of Animals with related codes of practice. Briefly, CIA was induced by intradermal injection at the tail base with 100 μg of bCII on day 0, boosted with an i.p. injection of 100 μg of bCII on day 21. This resulted in the onset of CIA between D30 –32 as described previously. Due to the expected broad viral tropism of the Ad5GFP-R7-knob or AdsGFP-FWT, gene transduction of RA FLS with a 40- to 50-fold higher efficiency than Ad5GFP-FWT, and in a dose-dependent manner at MOI between 20 and 200. These differences could not be attributed to differences in batch quality because A549 cells were permissive to both vectors (Fig. 2, A and B).

We next examined the dose-response curves of gene transduction of RA FLS by Ad5GFP-FWT-knob and Ad5GFP-FWT-knob, and in comparison with A549 cells (Fig. 2C). A549 cells were fully permissive to Ad5 infection and were used as a positive control for gene transduction (22). At least two different batches of each vector were used. AdsGFP-FWT-knob transduced RA FLS with a 40- to 50-fold higher efficiency than AdsGFP-FWT, and in a dose-dependent manner at MOI between 20 and 200. These differences could not be attributed to differences in batch quality because A549 cells were permissive to both vectors (Fig. 2, right panel). Consistent with previous studies (9, 14), FLS were found to be poorly permissive to Ad5 infection and were used as a positive control for gene transduction (22). At least two different batches of each vector were used. AdsGFP-FWT-knob transduced RA FLS with a 40- to 50-fold higher efficiency than AdsGFP-FWT-knob, and in a dose-dependent manner at MOI between 20 and 200. These differences could not be attributed to differences in batch quality because A549 cells were permissive to both vectors (Fig. 2C, right panel). Consistent with previous studies (9, 14), FLS were found to be poorly permissive to Ad5 infection and were used as a positive control for gene transduction (22).

The time course of Ad5GFP-R7-knob-mediated GFP gene transduction of RA FLS was examined over 24, 48, and 72 h at a MOI of 200. At all time points, AdsGFP-FWT-knob transduced RA FLS with a 40- to 50-fold higher efficiency than AdsGFP-FWT-knob (Fig. 2D).

**Effect of proinflammatory cytokines on gene transduction efficiency of RA FLS by Ad5GFP-R7-knob**

In vivo, FLS are exposed to proinflammatory cytokines such as IL-1 and TNF, which are key factors in the pathogenesis of RA. We next determined whether Ad-mediated gene transduction was influenced, positively or negatively, in the presence of these cytokines. FLS were pretreated with IL-1β (10 ng/ml) or TNF-α (10
Gene transduction efficiency of short fiber-modified Ad5 vectors compared with WT Ad5 in RA FLS. A, A representative fluorescent microscopic picture of GFP expression (green), double labeled with DAPI (blue) is shown in Ad5GFP-FiWT, Ad5GFP-R7-RGD, and Ad5GFP-R7-knob-infected RA FLS at a MOI of 200 PFU/cell at 48 h postinfection (p.i.). B, RA FLS were infected with Ad5GFP-FiWT, Ad5GFP-R7-RGD, and Ad5GFP-R7-knob at a MOI of 200 PFU/cell, and GFP-positive cells were counted at 48 h p.i. The results are expressed as the percentage of GFP-positive cells and represent the mean ± SEM of three separate experiments. *, p < 0.05 percent of GFP-positive cells transduced by Ad5GFP-R7-RGD or Ad5GFP-R7-knob compared with Ad5GFP-FiWT. C, Dose-response curves of Ad5GFP-FiWT and Ad5GFP-R7-knob vectors at MOI of 2 to 2000 PFU/cell for 48 h were examined in RA FLS and A549 control cells. The results are expressed as the percentage of GFP-positive cells and represented as the mean ± SEM of three separate experiments. *, p < 0.05 percent of GFP-positive cells transduced by Ad5GFP-R7-knob compared with Ad5GFP-FiWT.

ng/ml) for 48 h, then infected with Ad5GFP-R7-knob or Ad5GFP-FiWT at various MOI for 24 h. Ad5GFP-R7-knob-infected cells showed a 2-fold increase in gene transduction efficiency at a MOI of 20 for IL-1- or TNF-treated cells (Fig. 3). Furthermore, both fluorescence microscopy and flow cytometry showed a proportion of Ad5GFP-R7-knob-infected FLS, displaying a higher mean fluorescence intensity when pretreated with IL-1 or TNF (data not shown). In contrast, pretreatment with IL-1 or TNF had either no effect, or occasionally a negative effect, in Ad5GFP-FiWT-mediated transduction of FLS.

Role of fiber-knob domain in Ad5GFP-R7-knob attachment and entry of FLS

Because Ad5GFP-R7-knob most efficiently transduced RA FLS compared with RGD-ligated or conventional WT vectors, we next sought to identify the role of different capsid proteins in attachment of the virus to the cell surface and viral entry. The knob domain of Ad5 is known to bind other attachment receptors apart from CAR, which includes heparin sulfate glycosaminoglycans and the MHC class I α2 domain (23, 43, 44). We investigated the role of the knob domain in Ad5GFP-R7-knob entry of FLS by cell binding competition assays. RA FLS or control A549 cells were infected with Ad5GFP-R7-knob at a MOI of 200 in the presence or absence of increasing quantities of recombinant fiber-knob protein. Because the numbers of cell surface receptors for Ad5 is not known, the amounts of knob competitor represented the equivalent of 10²-, 10³-, and 10⁴-fold excess over the total number of knob protein present in the infectious virus inoculum. As shown in Fig. 4A (left panel), Ad5GFP-R7-knob-mediated gene transduction of RA FLS was not affected by competition with fiber-knob protein. In contrast, in control CAR-positive and integrin-rich A549 cells, Ad5GFP-R7-knob-mediated gene transduction was significantly reduced by competition with fiber-knob protein in a dose-dependent way, 2- to 5-fold with a 10⁴- to 10⁵-fold excess (Fig. 4A, right panel).

To confirm the lack of involvement of the fiber-knob domain in Ad5GFP-R7-knob transduction of RA FLS, we compared gene transduction between Ad5GFP-R7-knob and its knobless version Ad5GFPΔknob at a MOI of 2 and 20 (33), and anticipated that gene transduction would not differ between both vectors. Unexpectedly, we observed an increase in transduction of RA FLS by Ad5GFPΔknob, compared with Ad5GFP-R7-knob, with a 15-fold higher efficiency at a MOI of 2, and a 5-fold higher efficiency at a MOI of 20 (Fig. 4B). This suggested that not only was the fiber-knob domain not required by Ad5GFP-R7-knob in FLS attachment and infection, but that it may impede viral infection.

Role of viral capsid proteins hexon and Pb in Ad5GFP-R7-knob attachment and entry to FLS

We next examined whether other capsomers such as hexon or Pb were involved in Ad5GFP-R7-knob transduction of RA FLS by cell binding competition assays using increasing amounts of viral proteins as competitors. No competition effect was observed with hexon protein, even at amounts equivalent to a 100-fold excess over the theoretical numbers of hexon capsomers present in the virus inoculum (Fig. 4C). However, Ad5GFP-R7-knob gene transduction was significantly diminished in the presence of recombinant WT Pb protein, in

FIGURE 3. Effect of proinflammatory cytokines on Ad5GFP-FiWT and Ad5GFP-R7-knob GFP transduction in RA FLS. RA FLS were preincubated with IL-1 (10 ng/ml) or TNF (10 ng/ml) for 48 h, then infected with Ad5GFP-FiWT or Ad5GFP-R7-knob at varying MOI (2 to 2000 PFU/cell) for 24 h. Results are expressed as the percentage of GFP-positive cells and are represented as the mean ± SEM of three separate experiments. *, p < 0.05 percent of GFP-positive cells transduced by Ad5GFP-R7-knob at a MOI of 20 in the presence of either IL-1 or TNF compared with Ad5GFP-R7-knob at a MOI of 20 alone.
a dose-dependent way. WT Pb reduced Ad5GFP-R7-knob gene transduction by 41% at a 30-fold excess, and by 71% at a 300-fold excess over the Pb capsomers (Fig. 4C). This suggested that Pb protein, but not hexon, was involved in cell attachment and viral entry.

To confirm involvement of the Pb capsomers in Ad5GFP-R7-knob infection of FLS, we examined the effect of anti-Pb Ab on gene transfer mediated by Ad5GFP-R7-knob. RA FLS were infected with Ad5GFP-R7-knob at a MOI of 200 in the presence of anti-Pb antiserum or corresponding preimmune serum. Ad5GFP-R7-knob gene transduction was significantly reduced in a dose-dependent manner in the presence of anti-Pb Ab but not by preimmune serum. A 66% reduction was observed with 3 \( \times 10^{-4} \) g/ml, and 86% with 30 \( \times 10^{-4} \) g/ml anti-Pb Ab (Fig. 4D).

Ad5 endocytosis is known to be mediated by the binding of cellular integrins to specific recognition motifs on viral proteins, primarily RGD in Pb capsomers (5). The LDV sequence binds a different spectrum of integrins (36, 45). To identify which integrin binding sequences in the Pb capsomer were involved in Ad5GFP-R7-knob entry of RA FLS, we used Pb mutants in the RGD340 (R340E) or LDV288 (D288K) motif in cell binding competition experiments with Ad5GFP-R7-knob. When compared with WT Pb, Ad5GFP-R7-knob gene transduction was also reduced by R340E or D288K mutant, although only at high doses (i.e., at 300-fold excess) (Fig. 4E). This suggested that Ad5GFP-R7-knob used both RGD and LDV motifs, as well as additional peptide motifs of lesser-defined sequences (38), and hence a broader spectrum of integrins in attachment and entry of RA FLS.

Surface expression and role of integrin receptors in Ad5GFP-R7-knob infection of RA FLS

Because both RGD and non-RGD sequences were observed to be involved in Ad5GFP-R7-knob entry into RA FLS, we examined the expression of integrins on RA FLS, which may be potential
receptors for Ad5 by flow cytometry. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ bind to RGD sequences, whereas $\alpha_v\beta_1$ mediates binding to both RGD and non-RGD sequences, and $\alpha_v\beta_1$ binds to LDV motifs (5, 38). Consistent with the literature (10, 11, 46), $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v$, $\alpha_5$, and $\beta_1$ integrins were expressed in RA FLS as were $\alpha_v$ and $\beta_1$ (Fig. 5A).

We next examined the requirement for these integrin species in Ad5GFP-R7-knob infection of RA FLS by cell binding competition assays with mAbs against integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v$, $\alpha_5$, or $\beta_1$. RA FLS were infected with Ad5GFP-R7-knob at a MOI of 200 in the presence or absence of anti-integrin mAbs, and GFP-positive cells were analyzed after 24 h. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v$, and $\beta_1$, but not $\alpha_5$, participated in Ad5GFP-R7-knob-mediated gene transduction of FLS (Fig. 5B).

Effect of SF on Ad5GFP-R7-knob-mediated gene transduction

To investigate the clinical relevance of Ad5GFP-R7-knob as a vector in human RA, the effect of SF on gene transduction was examined. SF from 70% of RA patients are known to contain anti-Ad5-neutralizing Abs, which limit the efficacy of unmodified conventional Ad5 vectors in treatment of human disease (14, 47). To examine whether Ad5GFP-R7-knob remained efficacious in the presence of SF, even at a lower MOI compared with Ad5GFP-FiWT, Ad5GFP-R7-knob at a MOI of 200 was compared with Ad5GFP-FiWT at a MOI of 2000. Ad5GFP-FiWT and Ad5GFP-R7-knob at a MOI of 200 were compared with Ad5GFP-FiWT at a MOI of 2000. Vectors were preincubated with different dilutions of SF from six different RA patients for 1 h, then the mixture was added to RA FLS, and GFP expression was measured after 24 h. Ad5GFP-FiWT was completely neutralized by SF at 1/10, 1/100, and 1/1000 dilutions, even though it was used at a higher MOI of 2000 (Fig. 6A, left panel). In comparison, in the presence of SF at 1/10, 1/100, and 1/1000 dilutions, Ad5GFP-R7-knob at a MOI of 200 transduced 26–50 (mean, 36%), 33–60 (mean, 42%), and 30–53% (mean, 37%) of FLS, respectively (Fig. 6A, right panel). This corresponded to 58, 39, and 35% inhibition of Ad5GFP-R7-knob-mediated gene transfer in the presence of SF at dilutions of 1/10, 1/100, and 1/1000, respectively. Thus, in the absence of SF, infection by Ad5GFP-R7-knob was 40–50-fold higher in comparison to Ad5GFP-FiWT (refer to Fig. 2), and remained 35–40-fold higher in the presence of SF despite partial viral neutralization.

As a control, we examined the neutralizing effect of IgG-depleted SF vs undepleted SF on Ad5GFP-FiWT transduction of RA FLS (Fig. 6B). The IgG-depleted SF inhibited Ad5GFP-FiWT-mediated transgene expression significantly less compared with undepleted SF. We were able to confirm that the inhibitory effect of SF is almost entirely due to the presence of anti-Ad5 IgG Abs, as has been published previously (47). We similarly noted a slight inhibition of transgene expression, an effect that could not be attributed to cells or complement, because these components were expressed as the percentage of GFP-positive cells and represent the mean of three separate experiments. B, Ad5GFP-FiWT at a MOI of 2000 was incubated with SF from two representative samples of SF (patients 4 and 6) as described above, and results were expressed as the percentage of GFP-positive cells. C, Ad5GFP-FiWT at a MOI of 20 was incubated with SF from two representative samples of SF (patients 4 and 6) as described above, and results were expressed as the percentage of GFP-positive cells. D, Ad5GFP-FiWT at a MOI of 20–200 was incubated without SF (–SF) or with SF (+SF) from two representative samples of SF (patients 4 and 6) at a dilution of 1/10, then added to RA FLS. Results are expressed as the percentage of GFP-positive cells and are the mean of two experiments performed in duplicate.
We next investigated whether viral neutralization could be overcome by using increasing amounts of the most efficacious fiber-modified Ad5, Ad5GFPΔknob. Ad5GFPΔknob at a MOI of 20–200 was preincubated with SF from the same two representative RA patients (patients 4 and 6) at the lowest dilution (1/10), and RA FLS were infected as described above (Fig. 6D). In the presence of SF at 1/10, Ad5GFPΔknob transduction of RA FLS was inhibited by 54% at a MOI of 20, and 21% at a MOI of 50. At a higher MOI of 100 and 200, however, Ad5GFPΔknob-mediated gene transfer was not inhibited in the presence of SF.

**Gene transduction efficiency of short fiber-modified Ad5 compared with Ad5GFP-FWT in RA synovium explants**

To extend these studies to the actual joint situation, we examined gene transduction efficiency of Ad5GFP-R7-knob or Ad5GFPΔknob compared with Ad5GFP-FWT in an ex vivo synovium explant model. Freshly harvested 3-mm³ synovium pieces were placed in 6-well tissue culture plates in quadruplicate for 4 days, then infected with Ad5GFP-R7-knob, Ad5GFPΔknob, or Ad5GFP-FWT at a MOI of 200 for 24, 48, and 72 h. Explant tissue was infected at 4 days for investigation of infection efficiency of the vectors in both synovial explants and in explant outgrowth because it takes 3–4 days for cells

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**FIGURE 7.** Gene transduction efficiency of short fiber-modified vectors compared with Ad5GFP-FWT in RA synovium explants. Freshly harvested 3-mm³ synovium tissue pieces were placed in culture for 4 days then infected with Ad5GFP-FWT, Ad5GFP-R7-knob, or Ad5GFPΔknob at a MOI of 200 for 24, 48, and 72 h. GFP-positive cells in cellular outgrowth from synovium explant tissue (A and B, ×20) and in synovium explant tissue (C and D) were analyzed by fluorescence microscopy (left panels) and phase contrast microscopy (right panels) at low (C, ×20) and high magnification (D, ×100). Results are representative of at least three separate experiments in three different RA donors. E, Seventy-two hours p.i., synovial explants were fixed, paraffin embedded, and sections were examined for GFP expression by immunostaining using 3-amino 9-ethylcarbazole (red) and counterstained with hematoxylin; magnification, ×400. GFP expression was quantified in 10 high-power fields, averaged, and expressed as the number of GFP-positive cells/mm². *, p < 0.05.

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removed or inactivated before incubation with vectors, but might be due to incomplete depletion of IgG or possibly other inhibitory factors in the SF.

Anti-Ad5-neutralizing Abs from sera of patients with lung and liver cancer, and ascitic fluid from ovarian cancer patients have been demonstrated to be mainly directed against fiber and Pb capsomers (39, 48, 49). We investigated the hypothesis that anti-Ad5 Ab from SF of RA patients were primarily directed against both fiber and Pb, and not the knob protein, by examining the effect of SF on transduction of RA FLS by the knob-deleted vector Ad5GFPΔknob. Ad5GFPΔknob at an efficacious MOI of 20 was incubated with SF from two representative RA patients (patients 4 and 6) as described above, and GFP expression was measured at 24 h (Fig. 6C). In the presence of SF at 1/10, 1/100, and 1/1000 dilutions, Ad5GFPΔknob transduced a mean of 25, 37, and 40% of FLS. This corresponded to 76, 60, and 55% inhibition of Ad5GFPΔknob-mediated gene transfer, respectively. This suggested that the contribution of the fiber-knob domain in virus neutralization by SF was negligible, and that anti-Ad5 Abs in SF were mainly directed against capsid proteins and domains other than the fiber-knob, such as the Pb protein or the fiber shaft domain.
to migrate out of the tissue. There was no difference in the infection efficiency or rapidity of infection in synovial tissue, after immediate infection compared with 4 days later (n = 4 from four different RA donors; data not shown). GFP expression was determined by fluorescence microscopy. Control tissue showed minimal autofluorescence. Cellular outgrowth from synovium explants are FLS and have the characteristic spindle-like morphology (Fig. 7B) and flow cytometry findings consistent with standard measures of isolation and identification of FLS following synovial tissue digestion established in our laboratories and elsewhere, as described in Materials and Methods.

FLS from synovial outgrowth were transduced by Ad5GFP-R7-knob but not by Ad5GFP-FiWT (Fig. 7, A and B). FLS from synovial explants were grown to confluence and infected by short fiber-modified vectors compared with Ad5GFP-FiWT at a MOI of 200 and incubated with PE-conjugated CD14 or CD3. More than 90% of FLS cells and 23% of GFP+CD3+ cells in synovial lining were identified (Fig. 8A). Gene transfer was significantly higher in synovial macrophages and lymphocytes by Ad5GFP-R7-knob compared with Ad5GFP-FiWT at a MOI of 200 and incubated with PE-conjugated CD14 or CD3. More than 90% of FLS were transduced by Ad5GFP-R7-knob compared with 80% of GFP+CD14+ cells and 23% of GFP+CD3+ cells, and 1–10% of subtypes by Ad5GFP-FiWT (data not shown).

Rheumatoid synovium is characterized by chronic inflammatory cell infiltrates consisting mainly of macrophages, T lymphocytes, and resident FLS. Similar to FLS, human macrophages and lymphocytes are known to be mainly CAR negative (6). Reports in murine macrophage and lymphocyte cell lines show that these cell types are CAR negative, but αv integrin positive (3). To identify infected cell types in synovial tissue explants, double staining was performed for GFP+CD14+ or GFP+CD3+ cells by immunohistochemistry. In Ad5GFP-R7-knob-infected synovial tissue, GFP+CD14+ and GFP+CD3+ cells and GFP+ cells in synovial lining were identified (Fig. 8A). Gene transfer was significantly higher in synovial macrophages and lymphocytes by Ad5GFP-R7-knob compared with Ad5GFP-FiWT (Fig. 8B; p < 0.05). Ad5GFP-R7-knob or Ad5GFPΔknob transduced macrophages more efficiently than lymphocytes. RA synovial tissue was also enzyme digested, and whole cells were infected with Ad5GFP-R7-knob compared with Ad5GFP-FiWT at a MOI of 200 and incubated with PE-conjugated CD14 or CD3. More than 90% of FLS were transduced by Ad5GFP-R7-knob compared with 80% of GFP+CD14+ cells and 23% of GFP+CD3+ cells, and 1–10% of subtypes by Ad5GFP-FiWT (data not shown).

FIGURE 8. GFP expression in Ad5-transduced synovial macrophages and lymphocytes. Synovial explants infected with Ad5GFP-FiWT, Ad5GFP-R7-knob, or Ad5GFPΔknob at a MOI of 200 for 72 h were fixed, paraﬁn embedded, and sections were examined by immunostaining. A, Sections were double stained for GFP expression (red) and CD14 (blue) or CD3 (blue); magniﬁcation, ×200. B, GFP+CD14+ cells or GFP+CD3+ cells were quantiﬁed in 10 high-power ﬁelds, averaged, and expressed as the number of GFP-positive cells/mm2. *, p < 0.05.

FIGURE 9. Gene transduction efficiency of short ﬁber-modiﬁed vectors compared with Ad5GFP-FiWT in murine CIA. CIA was induced, and on day 32 control saline or 106 PFU/ml Ad5GFP-FiWT, Ad5GFP-R7-knob, or Ad5GFPΔknob were injected into both knee joints (n = 8 mice, 2 mice per vector group). Forty-eight hours after injection of the viruses, knee joints were ﬁxed, paraﬁn embedded, and decalcified. Frontal sections of the knee joints were examined for GFP expression (arrowheads) and counterstained with hematoxylin in inﬂamed knee joints. Control uninfected knee joint (top left panel), conventional Ad5GFP-FiWT-transduced knee joint (top right panel), Ad5GFP-R7-knob-transduced knee joint (bottom left panel), Ad5GFPΔknob-transduced knee joint (bottom right panel), b = bone, c = cartilage, = synovitis at a magniﬁcation of ×40 (A) and ×200 (B). C, A representative image of GFP-positive staining in Ad5GFPΔknob-infected murine synovium; magniﬁcation, ×400. D, GFP expression was analyzed in murine inﬂamed knee joints (left panel) and uninﬂamed knee joints (right panel). GFP expression was quantiﬁed in 10 high-power ﬁelds, averaged, and expressed as the number of GFP-positive cells/mm2. *, p < 0.05.
Gene transduction efficiency of short fiber-modified Ad5 compared with Ad5GFP-FiWT in murine CIA

Due to the expected broad viral tropism of the Ad5GFP-R7-knob or Ad5GFPΔknob vectors, targeted infection of synovial cells was achieved by i.a injection. To demonstrate efficacy of our short fiber-modified vectors even at a lower viral titer we used 1 × 10^6 PFU/ml, which is reduced compared with protocols used by other authors (1 × 10^7 PFU/ml), for conventional Ad5 vectors (13).

To determine whether transduction efficiency of short fiber-modified vectors was enhanced in the presence of inflammation in vivo, we examined transduction efficiency in inflamed knee joints in murine CIA. Macroscopic scores in the knees were no different between vector groups (1.5). Histological scores in frontal sections of the knees were likewise no different between vector groups (1.25–1.5). GFP expression was measured in mice with no evidence of arthritis at the time of i.a injection. To demonstrate efficacy of our short fiber-modified vector-infect synovial tissue. This was observed both throughout the synovium and at the pannus-cartilage junction. Gene transfer was significantly more efficient by Ad5GFP-R7-knob or Ad5GFP Δknob compared with Ad5GFP-FiWT in inflamed murine knee joints (Fig. 9D; p < 0.05).

We next examined in vivo transduction efficiency of the vectors in uninflamed knee joints in murine CIA. GFP expression was measured in mice with no evidence of arthritis at the time of i.a vector injection (n = 8 mice, two mice per vector group, one knee injected per mouse, macroscopic score 0). GFP expression was significantly higher in Ad5GFP-R7-knob or Ad5GFPΔknob-infected knee joints compared with Ad5GFP-FiWT-infected uninflamed knee joints (Fig. 9E; p < 0.05). Gene transfer by Ad5GFP-R7-knob, Ad5GFPΔknob was significantly higher in the presence of inflammation compared with uninflamed knee joints (2.8- and 2.5-fold higher, respectively). This is consistent with in vitro data in which proinflammatory cytokines enhanced transduction efficiency of short fiber-modified vectors. We also observed a trend to increased transduction efficiency of Ad5GFP-FiWT-infected knee joints (1.5-fold increase); however, this was not statistically significant. Similar to data in human synovium, in murine synovium there was no advantage of the knobless vector compared with Ad5GFP-R7-knob. Increased efficacy of the short fiber-modified vectors compared with Ad5GFP-FiWT is consistent with human in vitro and ex vivo data.

Discussion

Ad5 vectors have been extensively used in animal models of arthritis due to their ability to infect both proliferating and quiescent cells, ease of propagation, nonintegrating nature, and relative safety. However, both murine and human RA FLS have been reported to be poorly permissive to Ad5 vectors due to the absence of the high-affinity receptor CAR (9, 14). Although, low levels of transgene expression obtained with Ad5 can be compensated by the use of high MOI, this may induce toxicity and arthritis flares in murine models (2, 50). Furthermore, the high prevalence of anti-Ad5-neutralizing Abs in SF may limit the use of such vectors in human disease. Hence, strategies to improve Ad5 viral tropism would be desirable. In this study, we observed that genetically modified Ad5 vectors with short-shafted fibers (7 shaft repeats instead of 22 in the WT fiber) were highly efficient in transduction of RA FLS and human and murine synovium.

In RA FLS, this was enhanced by deletion of the knob domain and was more efficient than insertion of RGD motifs in the shaft. We demonstrated for the first time that in the context of the short fiber modification, Ad5 used a spectrum of both RGD and non-RGD-binding integrins to enhance viral tropism in RA FLS. In RA FLS, synovial macrophages, and lymphocytes, we observed that Ad5 vectors with short fibers were significantly more efficient in gene transduction than the conventional vector with WT fibers. This was more striking for RA FLS in which Ad5GFP-R7-knob was 40- to 50-fold, and Ad5GFP-R7-RGD 25-fold more efficient than Ad5GFP-FiWT. Furthermore, Ad5GFP-R7-knob more rapidly transduced cells within 24 h, compared with Ad5GFP-FiWT in both RA FLS and synovium. Finally, these vectors show promise in effective gene delivery even in the presence of neutralizing anti-Ad5 Abs in SF.

Shortened fiber length may be associated with enhanced viral tropism in CAR-deficient cells (19, 51, 52). Magnusson et al. (19) reported an increased viral tropism of Ad5GFP-R7-RGD in CAR-negative RD cells compared with Ad5GFP-FiWT. Subgroup B serotype Ad35 more efficiently transduced RA FLS compared with Ad5GFP-FiWT in RA FLS (14). Similarly, chimeric Ad5 vectors carrying subgroup B short fibers such as serotype 16 or 35 showed enhanced gene delivery to RA FLS compared with an Ad5 vector with WT fibers (9). Ad vectors with short fibers of <8 shaft repeats such as Ad9 also showed increased infection efficiency in CAR-deficient cells (51, 52). The authors (53) suggested that vectors with less than a critical number of shaft repeats may permit close proximity and accessibility of the viral Pb to integrin receptors on the cell surface. Integrin molecules protrude above the cell surface and are approximately the same height (53) as a fiber with a shaft of ~13.5 Å each. Integrin receptors could thus directly serve as both attachment and endocytic receptors by direct binding of the Pb. In CAR-deficient RA FLS, we observed that shortened fiber length of seven shaft repeats was a key determinant of Ad5 tropism and required integrin binding motifs in the Pb.

In assays to identify receptor usage, we observed that Ad5GFP-R7-knob attached to RA FLS in a knob-independent fashion. This was consistent with previous studies of Ad5GFP-R7-knob showing CAR-independent attachment and infection in other CAR-deficient cell types (19, 22). Furthermore, a deknobbed version of Ad5GFP-R7-knob, Ad5GFPΔknob lead to an unexpected increase in gene transduction in RA FLS compared with Ad5GFP-R7-knob. It has been suggested that steric hindrance provoked by the knob domain or repulsive acidic charges at the cellular surface may interfere with the infectivity of Ad5 vectors with short fiber shafts (27). This could be the case for Ad5GFP-R7-knob, and may explain the higher level of gene transduction observed with its knobless version, Ad5GFPΔknob.

Receptor specificity of the Pb is crucial in viral tropism (38, 54). The incorporation of the RGD motif into the HI loop or at the C-terminal end of the Ad5 fiber or hexon protein has previously been shown to enhance viral tropism in CAR-deficient cells, by retargeting the virus to cellular integrins (19–21, 55). We observed that enhanced transduction of RA FLS by Ad5GFP-R7-knob was knob-independent and did not exclusively occur via the RGD-dependent integrin pathway. The LDV motif and probably other less-defined sequences in the Pb may mediate binding to both α5β1 integrins and other species. This was suggested by cell-binding competition assays, in which the RGD motif of Pb had a significant competing effect, lower than WT Pb and trended to being less pronounced than LDV, although this was not statistically significant. This was also consistent with the lesser gene-transducing activity of Ad5GFP-R7-RGD, the RGD-ligated short-shafted vector compared with Ad5GFP-R7-knob. In addition, β1 integrin, which is the most highly expressed integrin on FLS, may bind both RGD and non-RGD motifs.
In the presence of IL-1 and TNF, Ad5GFP-R7-knob transduction of GFP was enhanced but was unchanged or at times reduced with Ad5GFP-FiWT. In addition, in cytokine-stimulated Ad5GFP-R7-knob-transduced cells, the mean fluorescence intensity of GFP was increased compared with unstimulated cells. This may be due to increased efficiency of postentry steps (9, 22). Similarly, in murine CIA we observed increased transduction efficiency of the short fiber-modified vectors in inflamed compared with uninfamed knee joints. This suggests that Ad5GFP-R7-knob and Ad5GFPΔknob-mediated gene delivery may be enhanced in inflammatory arthritis. Vectors up-regulated in the presence of inflammation may be advantageous due to the relapsing and remitting nature of RA. This concept has been extended to disease-regulated therapeutic transgene expression (56–58). Cytokines have been reported to down-regulate α integrins in RA FLs and synovium (12, 46). However, integrin expression levels do not correlate with Ad5-mediated transgene expression (13, 59). Increased efficacy of Ad5GFP-R7-knob, despite down-regulation of cellular integrins, could be attributed to an increased spectrum of integrin receptor choice and affinity for ligands other than RGD in our short-shafted vectors, rather than the absolute level of integrin expression. Ad5GFP-R7-knob or Ad5GFPΔknob more efficiently transduced both human and murine synovium compared with Ad5GFP-FiWT. In contrast to RA FLs, there was no advantage of the knobless vector compared with Ad5GFP-R7-knob. There was a trend to increased efficacy of Ad5GFP-R7-knob compared with Ad5GFPΔknob, although this was not statistically significant. The difference between transduction efficiency of these two vectors in RA FLs and synovial tissue may be explained by different integrin expression in FLs compared with cellular infiltrates in human or mouse synovium. RA FLs and tissue are known to express multiple integrins (10). Among these, RGD-binding integrins such as αβ1, αβ5, αβ6, non-RGD-binding integrins such as αβ1, and LDV-binding integrins αβ1 have been described (10, 12, 46). In murine synovial cells and tissue, CAR is also notably absent, and αβ5 and αβ6 integrins are expressed (13). Similarly, in murine lymphocyte and RAW macrophage cell lines, CAR is absent and, α integrins are expressed. In CAR-negative cells in synovium such as FLs, macrophages, and lymphocytes we observed increased transduction efficiency of the short fiber-modified vectors compared with the conventional vector. Thus, in the absence of CAR, several integrins may act together as coreceptors to increase viral tropism of Ad5GFP-R7-knob in RA. Thus, availability of Ad5 receptors may determine differential efficacy and selectivity of the short fiber-modified vectors. The differences in selectivity of Ad5GFP-R7-knob or Ad5GFPΔknob could be used advantageously depending on the cellular target in gene therapy. It could be envisaged that Ad5GFPΔknob may be used predominantly in targeting RA FLs for delivery of proapoptotic therapies in which a “genetic synovectomy” effect is desired. In contrast, more broad-spectrum anti-inflammatory effects may be desirable by use of either Ad5GFP-R7-knob or Ad5GFPΔknob in delivery of anti-inflammatory therapies.

Immunogenicity due to the high prevalence of anti-Ad5 Abs may decrease efficiency and feasibility of Ad5 vectors in the clinical setting. This major disadvantage has prompted use of other Ad serotypes such as Ad35, chimeric Ad5 vectors, or alternative viral vectors such as retroviral or adenovectorial vectors in gene therapy of arthritis (3, 14, 47). As much as 70% of RA patients contain neutralizing anti-Ad5 Ab in the SF (47). We observed that Ad5GFP-R7-knob maintained up to a 40-fold increase in gene transduction compared with Ad5GFP-FiWT in the presence of SF. In contrast, Ad5GFP-FiWT was completely neutralized. Furthermore, Ad5GFPΔknob was also partially neutralized in the presence of SF at low MOI of 20–50, suggesting that a significant proportion of anti-Ad5-neutralizing Abs were directed against the Pb and possibly fiber shaft, and not the knob domain. However, at higher MOI of 100–200, Ad5GFPΔknob completely over-rode the neutralizing effects of SF. This suggests that epitopes in the native Pb, which are crucial for Ad5 infection, may be different from those targeted by neutralizing Ad5 Abs, and may have a higher affinity for integrin receptors. This may be consistent with a previous study demonstrating that RGD-specific Ad5 Abs were not promiscous in human subjects and had weak neutralizing activity, whereas other Pb epitopes were found to be highly neutralizing (48).

We attempted to address the question of whether pre-existing immunity against Ad5 may affect transgene duration in human in vitro studies, because we believe this is more relevant to preclinical human applications. We have shown that there is a clear advantage in use of the short fiber-modified vectors over the conventional WT in avoidance of viral neutralization. Our observation that short fiber-modified vectors circumvent viral neutralization suggests they should have a significant advantage in duration of transgene expression in the human context. In the absence of viral neutralization, Ad5 transgene expression may persist for as long as 6 wk (1, 2). It would be interesting to determine whether the short fiber-modified vectors induced a reduced immune response (Ab formation) against the virus and more prolonged transgene expression in vivo. We are currently generating therapeutic vectors, and in our next study we will examine the question of prolonged therapeutic benefit of the vectors and immune response, which we believe would be more relevant to clinical applications.

In conclusion, Ad5GFP-R7-knob and its deknobbed version Ad5GFPΔknob are two potential powerful novel vectors for local i.a gene delivery in human and murine arthritis. Key determinants of Ad5 tropism in RA FLs include shortened viral fiber length and receptor specificity of the Pb to a spectrum of non-RGD and RGD-binding integrins. Furthermore, knob ablation further enhanced efficacy in RA FLs, which in turn would reduce the required vector dose and potential for induction of arthritis flares. In addition, in the event of even minimal leakage of the vector into the systemic circulation postarticular injection, knob ablation would detarget the vector from CAR-positive liver cells and hence reduce potential liver toxicity. In contrast to the previous focus on altering the knob domain in retargeting strategies, tropism of the native Pb may optimize use of integrins as both attachment and endocytic receptors to enhance gene transduction in RA FLs. An additional advantage of highly efficacious gene transfer using Ad5GFPΔknob was the ability to completely circumvent viral neutralization by SF. Use of such fiber-modified Ad5 may revive the potential clinical usefulness of Ad5 vectors in gene therapy of human RA patients. Use of these vectors in delivery of a therapeutic transgene would be highly desirable, and work has commenced to examine the functional sequelae of the improved constructs compared with the conventional WT in our in vitro, ex vivo, and in vivo models targeting IL-17.

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