

# Characterization of Adenovirus-Mediated Gene Transfer in Rabbit Flexor Tendons

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**Purpose:** Adenoviral vector-based gene therapy is a promising technique for the delivery of growth factors to tendons. The objective of this study was to determine whether rabbit flexor tendons could be transduced effectively by adenoviral vectors and whether the introduction of adenoviral vectors would cause a notable local inflammatory response.

**Methods:** Recombinant adenoviruses expressing green fluorescent protein (AdGFP) or BMP-13 (AdBMP-13) were constructed and 3 different viral titers ( $1 \times 10^7$ ,  $1 \times 10^8$ , and  $1 \times 10^9$ ) were tested in this study. The second through fifth tendons of the forepaws and hindpaws of a New Zealand white rabbit were identified surgically and injected with different viral titers of adenoviruses. The fifth tendon was used as a control. The tendons were harvested 12 days after surgery. The retrieved tendons were sectioned to measure transgene expression, as well as for histologic evaluation.

**Results:** At all tested viral titers an efficient dose-dependent transgene expression was detected in all samples at 12 days after injection. At the highest dose the injection sites were notable for lymphocytic infiltration, suggesting that injected adenoviral vectors can illicit some local inflammatory response. Lymphocytic infiltration was much less apparent, however, in the tendons injected with lower titers of adenoviral vectors. There was no evidence of a massive inflammatory response and/or cell death.

**Conclusions:** Our findings show that adenovirus-based gene therapy is an efficient means of gene delivery to rabbit flexor tendons. Transduction efficiency of transgenes was dose dependent across the tested titers, although adenovirus-induced inflammation was notable only at the highest titer. This indicates that efficient gene transfer without notable local inflammatory response may be achieved by using the lower titers. Although adenovirus-induced inflammation can be minimized by using lower viral titers, its impact on adhesion formation in the long term remains unknown. (*J Hand Surg* 2005; 30A:136-141. Copyright © 2005 by the American Society for Surgery of the Hand.)

**Key words:** Adenoviral vector, bone morphogenetic protein (BMP), flexor tendon, gene therapy, inflammation.

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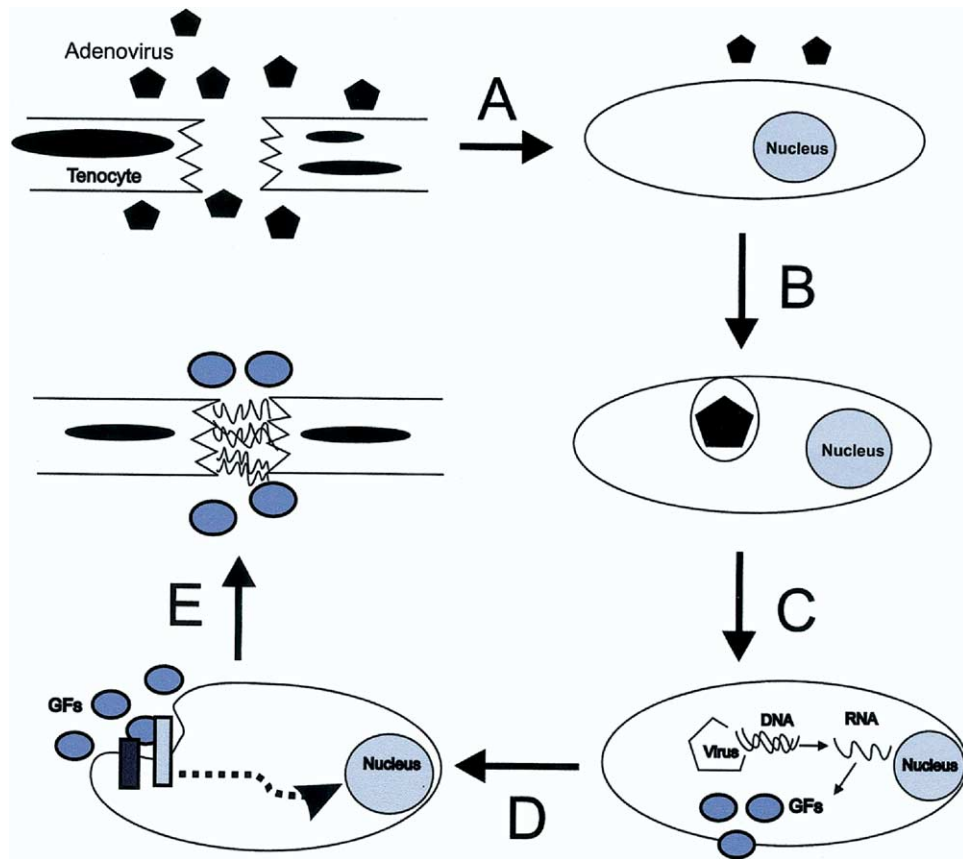
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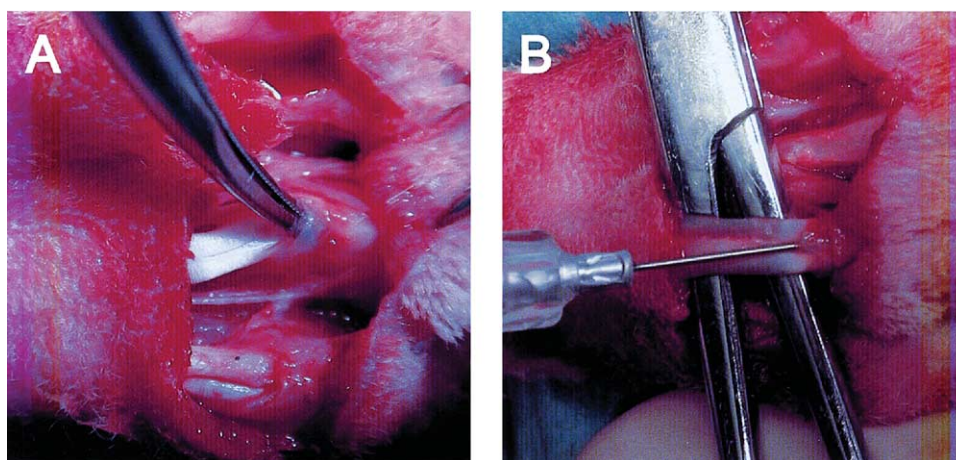


**Figure 1.** The use of gene therapy in tendon repair. (A) The adenovirus vectors expressing growth factors (such as BMPs) are delivered to the site of tendon laceration. (B) The viruses attach to the cell membrane of the tenocyte and next (C) enter the cytoplasm. (D) The DNAs encoding for the growth factors are transcribed to RNA and then translated to proteins (GFs). (E) The tenocyte then secretes the factors into the extracellular environment where they can bind to receptors on nearby cells and promote tendon healing.

Flexor tendon lacerations represent a common yet challenging problem. Outcomes of surgical treatment often are compromised by rupture or adhesions.<sup>1,2</sup> Attempts to decrease the rupture rate, such as immobilization or bulky suture technique, often increase adhesions.<sup>3</sup> Conversely attempts to decrease adhesions, such as early active range of motion, often lead to tendon rupture.<sup>4,5</sup> Recently biologic augmentation of flexor tendon healing has been explored as a solution to this dilemma. If the biology of flexor tendon healing can be altered to provide a stronger repair, then earlier and more aggressive therapy potentially could be started. This in turn would lead to fewer adhesions with the end result being a more functional finger.

Attempts to influence the biologic milieu in favor of improved tendon healing has attracted a lot of attention,<sup>6-9</sup> particularly with respect to the bone morphogenetic proteins (BMPs).<sup>10,11</sup> The use of gene therapy to deliver BMPs results in sustained expression of a selected transgene and may

be superior to recombinant protein with respect to biological activity and longevity of effect (Fig. 1). We previously showed transgene expression of up to 6 weeks in an *in vitro* tenocyte model (unpublished data). Successful *in vivo* transfection of tendons via adenoviral-based gene therapy also has been shown.<sup>12,13</sup> The collective experience with gene therapy in flexor tendons, however, remains limited. Adenoviral delivery of BMP-12 to a chicken flexor tendon *in vivo* has been successful; however, the optimal viral delivery titer remains elusive.<sup>11</sup> The use of higher titers may provide better rates of transfection but it may cause notable inflammation, which in turn causes tendon adhesions.<sup>14-16</sup> Flexor tendons are relatively immunoprivileged sites and therefore may be less prone to adenovirus-induced inflammation. In this study we used an *in vivo* rabbit flexor tendon model to determine the optimal viral titer for efficient gene transfer with minimal local inflammatory response.



**Figure 2.** Delivery of adenovirus to tendon. (A) The entrance of the tendon sheath is identified. (B) The adenovirus is injected under direct visualization into the flexor tendon using a 27-gauge needle.

## Materials and Methods

### Construction of Recombinant Adenoviruses

The complementary DNA clone for human BMP-13 (also known as growth differentiation factor-6 or cartilage-derived morphogenetic protein-2) was kindly provided by the Genetics Institute (Cambridge, MA). The coding region of the BMP-13 then was sub-cloned into the pAdTrack-CMV shuttle vector. The resultant pAdTrack BMP-13 was used to generate adenoviral recombinants using the AdEasy technology as previously described.<sup>17</sup> The adenoviral recombinants were used to produce adenovirus in HEK 293 packaging cells, resulting in an AdBMP-13 adenoviral vector that also contained a built-in green fluorescent protein (GFP) expression cassette. A recombinant adenoviral vector expressing GFP alone also was constructed as a control vector (AdGFP).

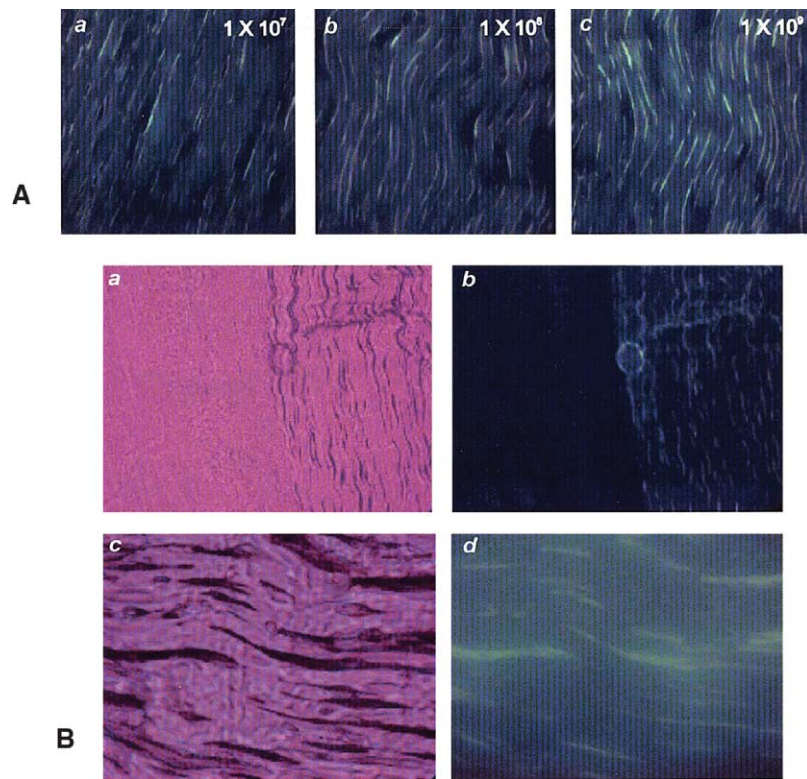
### Direct Injection of Recombinant Adenoviral Vectors Into Rabbit Flexor Tendons

The use and care of New Zealand white rabbits in the experiment was approved by the Institutional Animal Care and Use Committee. After the induction of anesthesia to a single rabbit, the bilateral forepaw and hindpaw were shaved and prepped. By using a tourniquet and sterile technique an L-shaped incision was made over the palmar aspect of the right forepaw and hindpaw. The second through fifth flexor profundus tendons were identified as they entered the flexor tendon sheaths. As shown in Figure 2, on each paw the second tendon was injected under direct visualization by using a 27-gauge needle with 10  $\mu\text{L}$  (containing  $1 \times 10^7$  plaque forming units [pfu]) of AdBMP-13. The needle was inserted into the proximal entrance of the tendon sheath, thereby avoiding the need for sheath

incision or repair. Subsequently the third tendon was injected with 10  $\mu\text{L}$  (containing  $1 \times 10^8$  pfu) of AdBMP-13 and the fourth tendon was injected with 10  $\mu\text{L}$  (containing  $1 \times 10^9$  pfu) of AdBMP-13. The fifth tendon was left untouched to serve as a control. The same procedure was performed on the left forepaw and hindpaw by using the same titers and tendons but now with AdGFP. In this manner the inflammation caused by the adenovirus can be differentiated from any possible inflammation caused by BMP-13. The skin then was closed with 4-0 nylon sutures. The paws were wrapped with a nonadherent dressing, gauze, and an elastic bandage. The rabbit was allowed to walk freely in its cage and was given free access to food and water. The rabbit was monitored closely after surgery. Pain was well controlled with buprenorphine (20–50  $\mu\text{g}/\text{kg}$ ) as needed. The tendons were harvested on day 12 after surgery, embedded in an optimum cutting temperature compound, and frozen in liquid nitrogen.

### Histologic Evaluation and Transgene Expression

Frozen sections were prepared with a microtome (HM550 Cryostats, Richard-Allen Scientific, Kalamazoo, MI), each measuring 10  $\mu\text{m}$  in thickness. Frozen sections then either were stained with hematoxylin-eosin or were evaluated under a fluorescence microscope for GFP expression. As described in the vector construction, the adenoviral vector AdBMP-13 also contained a GFP expression cassette. In this manner the GFP reporter is co-expressed along with the transgene. This configuration allowed us to track transgene expression effectively under a fluorescent microscope. In this



**Figure 3.** Efficiency of transgene expression in the harvested tendons. (A) All 3 titers showed strong green fluorescence, indicating efficient transgene expression (fluorescence microscopy of fresh-frozen sections; magnification, x300). A dose-dependent gradient can be observed with less fluorescence in the  $1 \times 10^7$  pfu (a) and more fluorescence in the  $1 \times 10^9$  pfu group (c). (B) Photomicrographs of an injected tendon that includes both the injection site with infected tenocytes as well as uninfected tenocytes in the surrounding tendon are shown using hematoxylin-eosin bright field microscopy (a and c) versus fluorescence microscopy (b and d) at different magnifications: x100 (a and b) or x300 (c and d). Transgene expression is confined to tenocytes and does not occur in surrounding uninfected tendon. Representative samples infected with AdBMP-13 are shown.

experiment the tendons were sectioned parallel to the fibers of the tendon and evaluated for GFP expression.

### Inflammatory Changes in the Flexor Tendons

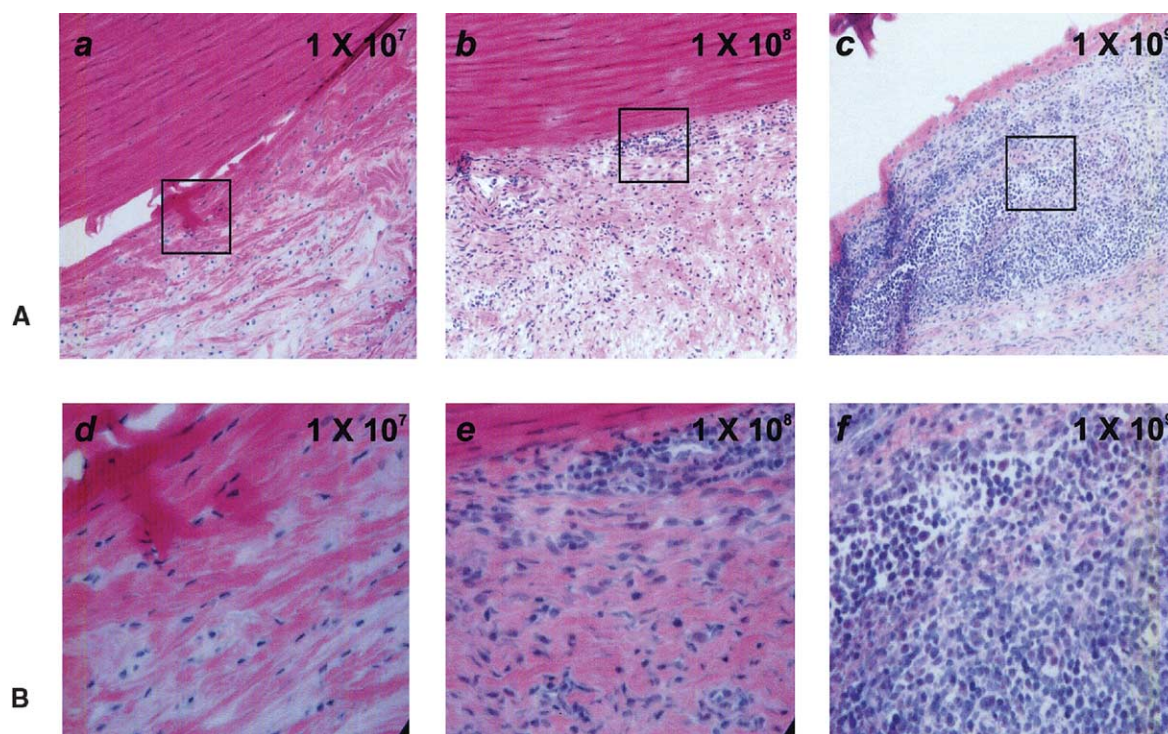
Frozen sections for each tendon were prepared as previously described. These were stained with hematoxylin-eosin and examined with light microscopy. The presence of inflammation for each viral titer was determined and compared. The difference in inflammation between the AdGFP and AdBMP-13 groups also was examined and compared. This was performed to differentiate the inflammation caused by the adenovirus from any possible inflammation caused by BMP-13.

## Results

### AdBMP-13 Efficiently Transfects Tenocytes in an *In Vivo* Flexor Tendon Model

To assess whether tenocytes can be transduced efficiently with AdBMP-13 or AdGFP, we used an *in*

*vivo* direct injection model into rabbit flexor tendons. Expression of the GFP reporter was detected readily at 12 days after surgery, indicating successful transfection of all tendons. The signal was robust and showed a dose-dependent gradient (Fig. 3). As opposed to previous studies,<sup>18</sup> we found that the transgene expression was detectable throughout the flexor tendon and was not limited to the injection sites. There was no difference in transgene expression between the AdBMP-13 and AdGFP groups, suggesting that the efficiency of transfection may be independent of transgenes. All 4 control tendons, which were not exposed intentionally to AdGFP or AdBMP-13, also showed signs of transgene expression. The control tendons were located next to the tendon receiving the highest titer,  $1 \times 10^9$  pfu, on each paw. The transgene expression in the control tendons may have been caused by local spillage, and nevertheless was testament to the transfection efficiency of the adenoviral vectors.



**Figure 4.** Inflammatory changes are present in the harvested tendons infected with high titer of adenoviruses. Photomicrographs of tendons show a lymphocytic infiltration of the tissue surrounding the synovium (hematoxylin-eosin): (A)  $\times 100$  and (B)  $\times 300$ . A dose-dependent effect can be seen with the highest titer,  $1 \times 10^9$  pfu (c and f), showing the most inflammation. Representative samples infected with AdBMP-13 are shown.

### Adenovirus-Induced Dose-Dependent Inflammatory Changes in the Flexor Tendons

To assess the extent of adenoviral- or transgene (GFP or BMP-13)-induced inflammation, histologic examination of hematoxylin-eosin-stained sections was performed. The inflammation, as shown by the presence of inflammatory cells, was compared across titer levels as well as between AdGFP and AdBMP-13 groups. Inflammatory changes were found in all 3 viral titers. Furthermore the inflammation increased with the amount of virus delivered in a dose-dependent fashion (Fig. 4). The inflammation consisted mostly of lymphocytes in the tissue surrounding the tendons. No difference was noted between the GFP or BMP-13 groups. No loss of transgene expression or cell death was observed during the course of the study.

### Discussion

The manipulation of the biologic milieu during flexor tendon healing may lead to better clinical outcomes. This can be accomplished by increasing the flexor tendon strength or by decreasing adhesion formation. BMPs have been shown to improve the tensile

strength of healing tendons.<sup>10,11,19</sup> To be most effective, BMPs ideally would be delivered in a way that would allow them to be present throughout the healing process. Gene therapy is one such way by which BMPs could be delivered to a flexor tendon and expressed for a prolonged period of time. Adenoviruses are ideal gene delivery systems for this application given that they are replication-defective forms of benign viral agents. They have a broad tropism and can infect nondividing cells, unlike many other viral vectors. They are not incorporated in the genome, which decreases the occurrence of insertional mutations; however, as a result transgene expression often does not persist beyond 4 to 6 weeks. Perhaps the single-most concerning attribute of adenoviruses, particularly with respect to intrasynovial applications, concerns their immunogenicity. Characterizing inflammation after the use of adenovirus-mediated transgene expression therefore was one of the main goals of this study.

It is clear from this and other studies that the transfection rate is dose-dependent.<sup>20</sup> It also is evident that excessive viral titers can lead to inflammation, loss of transgene expression, and even cell death in certain tissue types. The most vexing

problem after flexor tendon repair is the formation of adhesions. Adhesion formation is correlated directly with inflammation and therefore adenovirus-induced inflammation becomes of paramount importance in the flexor tendon. The flexor tendon itself is a relatively immunoprivileged site and may be less susceptible to adenovirus-induced inflammation than other tissues. This study reveals that at postoperative day 12, there is a dose-dependent inflammatory response to all 3 viral titers. This response does not appear to cause loss of transgene expression or cell death in the titers studied. The amount of inflammation is similar between the GFP and BMP-13 groups, suggesting the inflammation is secondary to adenovirus and independent of the gene being delivered. Although no obvious detrimental effects of the inflammation were noted in this study it is a cause for concern. Adenoviral-induced inflammation may lead to adhesion formation, decreased range of motion, and poor clinical outcome. The current study is a short-term evaluation of adenovirus-induced inflammation in flexor tendon and the importance of viral titer on modulating this effect. It remains to be seen in longer-term studies whether inflammation remains low for the duration of tendon healing, and whether lower titers of virus also decrease the risk for adhesion formation within the tendon sheath.

A possible limitation to this study is the potential for pseudofluorescence being mistaken for true fluorescence. This was of particular concern with the unexpected finding of fluorescence in the control tendons. To further examine this possibility the fields containing both the tendon and surrounding tissue were examined for fluorescence. If pseudofluorescence were present it would be seen throughout all tissues in the field; however, this was not encountered (Fig. 3). The correlation of fluorescence and cell morphology in the flexor tendons also makes it unlikely that pseudofluorescence is present.

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