NON-INVASIVE ADMINISTRATION OF ADENO-ASSOCIATED VIRAL VECTORS

INTRODUCTION

Background

Adeno-associated viruses (AAV's) were discovered as satellite viruses present in preparations of adenovirus. AAV isolates (human, simian and avian) cause no apparent disease and are not associated with cancer or any other adverse effects. Several studies have shown AAV to have a protective effect on carcinogenesis mediated by other viruses, such as tumorigenic strains of adenovirus and bovine papilloma virus.

Recombinant AAV vectors have been used to express gene products in vitro (usually tissue cultures) and to express gene products in animals, see, for example, U.S. Pat. No. 5,193,941 and WO 9413788, usually in the lungs or oral mucosa, the normal sites of AAV infection, but also in the central nervous system and in cardiac tissue.

Transduction with recombinant AAV vectors has been accomplished with more direct routes of administration, such as, intraperitoneal injection, intratracheal injection and direct endobronchial instillation in animals, see, for example, Flotte and

Carter, Gene Therapy 2:357-362 (1995). Those methods of delivering rAAV were focussed on situating the recombinant AAV vector at a site closest to the lung as the experiments were directed to treating patients with cystic fibrosis. However, it is well established that intraperitoneal, intratracheal and endobronchial injection generally are undesirable clinical techniques for routine use in humans, for example, due to the dangers of producing infection and adhesions, see Goodman and Gilman, "The Pharmacological Basis of Therapeutics" pp. 1-32 (8th ed., 1993); see also "Remington's Pharmaceutical Sciences" p. 1686 (18th ed., 1990).

One goal of gene therapy is to provide long-term, therapeutically effective levels of expression of a particular gene of interest in or adjacent to the tissues where such gene expression is therapeutically beneficial. There are many challenges to the development of effective gene therapy, including obtaining expression of the gene of interest in the targeted tissues, which requires at least a promoter in the recombinant AAV vector that operates in the targeted tissues, and a mode of administration that delivers the recombinant AAV vector to the appropriate tissues.

Accordingly, there is a need in the art for safe

and effective methods of administering recombinant AAV vectors for gene therapy.

SUMMARY OF THE INVENTION

The instant invention provides new methods of administering recombinant AAV vectors for effective, long-term expression of a gene of interest in animals, including humans. Provided herein are methods of subcutaneously injecting recombinant AAV vectors containing a gene of interest encoding any diffusible polypeptide, ribozyme, nucleic acid or antisense oligonucleotide for gene therapy. The instant invention also provides for a topical administration means of rAAV to a host.

The methods of the instant invention provide significant advantages, including the ability to deliver any suitable gene of interest via subcutaneous injection or topical means and obtain high level, long-term expression in regions approximate or distant from the site of administration. The administration routes of the instant invention provide a safe, effective and non-invasive procedure for the administration of recombinant AAV vectors as described herein.

The discovery that subcutaneous injection of recombinant AAV vectors for gene therapy is effective

and provides high-level, sustained expression is surprising, especially in light of the normal association of AAV with the lungs and oral passages, the requirement that promoters for recombinant AAV vectors be operable in the tissues targeted for gene expression and the dilution effect of subcutaneous injection, which typically results in rapid drainage of subcutaneously injected recombinant AAV vectors into the lymph nodes and rapid clearance by the host immune system.

The methods and recombinant AAV vectors described herein provide a significant development in the field of recombinant AAV vector gene therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the rAAV-MFG-human-Factor IX vector. ITR: AAV inverted terminal repeat; MFG: murine Moloney virus long terminal repeat; MuLV IVS: mRNA splice donor/splice acceptor; Human FIX: human Factor IX cDNA; bGH pA: bovine growth hormone polyadenylation site.

FIG. 2 is a schematic representation of the rAAV-MD-human-interferon vector. ITR: AAV inverted terminal repeat; CMV: Cytomegalovirus immediate early promoter; β-gb IVS: β-globin mRNA splice donor/splice acceptor; Human IFN: human interferon cDNA; β-gb pA:

β-globin polyadenylation site.

FIG. 3 is a schematic representation of the rAAV-MD-mouse erythropoietin vector. ITR: AAV inverted terminal repeat; CMV: Cytomegalovirus immediate early promoter; β-gb IVS: β-globin mRNA splice donor/splice acceptor; Mouse EPO: mouse erythropoietin cDNA; β-gb pA: β-globin polyadenylation site.

FIG. 4 is a schematic representation of the rAAV-CMV-LacZ vector. ITR: AAV inverted terminal Repeat; CMV: Cytomegalovirus immediate early promoter; SV40 IVS: SV40 mRNA splice donor/splice acceptor; LacZ: E. coli ß-galactosidase cDNA; SV40 pA: SV40 polyadenylation site.

FIG. 5 is a graph showing long-term expression of human Factor IX in immunocompetent mice that were injected subcutaneously with 5.9×10^{10} particles of rAAV-MFG-hFactor IX.

FIG. 6 is a graph showing long-term expression of human Factor IX in immunocompetent mice that were injected subcutaneously with 5.9×10^{9} particles of rAAV-MFG-hFactor IX.

FIG. 7 is a graph showing long-term expression of human Factor IX in immunocompetent mice that were injected subcutaneously with 5.9×10^{8} particles of rAAV-MFG-hFactor IX.

FIGS. 8A and 8B are two charts showing anti-human Factor IX antibody levels and anti-AAV antibody levels, respectively, in animals subcutaneously injected with a single injection of particles of rAAV-MFG-hFactor IX. FIG. 8A depicts antibody titers for anti-FIX activity. FIG. 8B shows antibody titers for anti-AAV activity.

FIGS. 9A and 9B depict long-term expression of human Factor IX in immunocompetent (BALB/c) and immunocompromised (NIH-3) mice after subcutaneous injection with recombinant AAV-MFG-human Factor IX (each injection contained 5.9×10^{10}, 5.9×10^{9} or 5.9×10^{8} particles).

FIG. 10 illustrates the anti-human Factor IX antibody levels of the animals described in FIG. 9.

FIGS. 11A and 11B depict long-term expression of human interferon in immunocompetent (BALB/c) and immunocompromised (NIH-3) mice after subcutaneous injection with recombinant AAV-MD-interferon (at 8.5×10^{10}, 8.5×10^{9} or 8.5×10^{8} particles).

FIG. 12 is a chart illustrating expression of mouse EPO in BALB/c mice following subcutaneous injection of rAAV-MD-mouse-EPO (8.9×10^{9} or 8.9×10^{10} particles). The expression of mouse EPO was measured by monitoring the hematocrit.

FIG. 13 Epo protein levels at day 105 following

SC injection of rAAV-MD-Epo. The protein levels were measured by RIA.

FIGS. 14A and 14B depict the hematocrit of BALB/c mice after subcutaneous or intramuscular injection of rAAV-MD-mouse-Erythropoietin.

FIG. 15 Epo protein levels at day 180 following subcutaneous or intramuscular injection of rAAV-MD-Epo. Protein levels were measured by RIA.

FIG. 16 is a chart illustrating human interferon levels in the serum of BALB/c mice after intravenous injection of AAV-MD-human IFN.

FIG. 17 is a chart illustrating human Factor IX levels in the serum of BALB/c mice after intramuscular injection of rAAV-MFG-F9.

FIG. 18 ß-galactosidase expression was not detected in the panniculus carnosus of uninfected animals.

FIG. 19 ß-galactosidase expression in the panniculus carnosus following subcutaneous injection of rAAV-CMV-LacZ.

FIG. 20 is a diagram of pSSV9 MD-2 AAV expression vector.

FIG. 21 is a diagram of the SSV9 MD2-mEPO AAV vector.

FIG. 22 is a diagram of the SSV9 MD2-human alpha 2A interferon vector.

FIG. 23 is a diagram of the SSV9-MFG-ShuF9 vector.

FIG. 24 is a diagram of the MFG-S-hFIX vector.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Provided herein are methods of subcutaneously injecting or topically applying recombinant AAV vectors containing a foreign gene of interest encoding any diffusible polypeptide, ribozyme, nucleic acid or antisense polynucleotide for gene therapy. The methods of the instant invention provide significant advantages, including the ability to deliver any diffusible gene of interest via subcutaneous injection or topical application to obtain high level, long-term expression in regions proximal or distal from the site of administration.

For example, human Factor IX was expressed in high levels in approximately 2/3 of the immunocompetent mice subcutaneously injected with a recombinant AAV vector containing the human Factor IX gene. The immunocompetent mice that expressed human Factor IX contained high serum levels of human Factor IX for about 365 days after a single injection (see FIG. 6). Subcutaneous injection of recombinant AAV vectors containing the human interferon gene or the mouse erythropoietin gene also resulted in high

serum levels of both gene products, lasting at least 175 days and 35 days after subcutaneous injection, respectively.

Due to the high level, long-term expression obtained with the methods and recombinant AAV vectors described herein, repeat dosages do not need to be frequent and can be administered at periodic intervals. Subcutaneous injection or topical application for gene therapy is a simple, safe and non-invasive method of administering the recombinant AAV vectors, which is much preferred to other, more invasive methods of administration described previously (e.g., intraperitoneal, intrathecal and endobronchial).

Subcutaneous or topical delivery of recombinant AAV vectors also provides the advantages of ease of handling and administration (e.g. self administration). Subcutaneous injection of recombinant AAV vector particles enables immunocompetent animals to express high levels of the gene of interest over a long term, whereas other routes of delivery of certain recombinant AAV vector particles do not result in comparable levels of expression, compare FIGS. 12, 16 and 17.

Expression of the gene of interest in immunocompromised animals was found to be

dose-dependent and consistent. Expression was found to increase within the time periods tested. Moreover, delivery of a syngeneic gene, like mouse erythropoietin, injected via a recombinant AAV vector in immunocompetent mice, resulted in dose-dependent, high level and consistent expression, see FIG. 12.

The recombinant vectors of the instant invention comprise the following components: (1) a promoter derived from a non-AAV source, (2) an mRNA splice donor/splice acceptor, (3) a gene of interest, for example, that encodes a therapeutic polypeptide or a therapeutically effective portion thereof, (4) a polyadenylation site and (5) two AAV inverted terminal repeats flanking components (1)-(4) above.

The gene of interest can be any polynucleotide that encodes a diffusible polypeptide, i.e. a polypeptide that can diffuse from the site of administration to effect systemic delivery, or a diffusible nucleic acid. For example, diffusible polypeptides that may be useful for gene therapy include insulin, Factor VIII and any cytokines, including interferons (IFN-α, IFN-β and IFN-γ), interleukins, GM-CSF (granulocyte-macrophage colony-stimulating factor), M-CSF (macrophage colony-stimulating factor), tumor necrosis factors and growth factors (TGF-β (transforming growth factor-β)

and PDGF (platelet-derived growth factor)).

It will be readily apparent to one of ordinary skill in the art that the methods described herein can be used to express, via subcutaneous injection or topical administration, any diffusible polypeptides of therapeutic interest. The gene of interest also may be an antisense polynucleotide or ribozyme.

The promoter can be any of the many characterized viral (e.g., thymidine kinase and Rous sarcoma virus) or cellular promoters (e.g. neural-specific enolase, vimentin and fibronectin). The accompanying regulatory signals also can be used for expression of transgenes in the instant vector backbone. Regulated gene expression from an inducible promoter (e.g. metallothionein etc.) also is capable of directing expression of transgenes in the instant recombinant AAV vectors. CMV expression cassettes also can be used for controlled gene expression.

Described herein are investigations regarding simple, non-invasive procedures for direct in vivo administration of recombinant AAV vectors encoding secreted therapeutic foreign gene products. Recombinant AAV vector particles encoding human Factor IX (huFIX) or human α-interferon (huIFN) were prepared and injected under the skin of immunocompetent BALB/c mice (10^8 to 10^{10} particles in a 300 μl volume of HBSS

(Hank's Balanced Salt Solution)). The presence of the secreted proteins was monitored in serum samples collected over time after a single subcutaneous injection.

Using either vector, a durable systemic release of the human proteins was observed reproducibly. Typically, levels of 5-50 ng/ml huFIX and 2-7 ng/ml huIFN were attained over a period of several weeks. The levels remained constant or increased slightly until the latest available time point. The situation typically was observed in 2 or 3 animals within experimental groups of 5.

When the experiments were repeated in fully immunodeficient NIH-3 mice, long-term expression was obtained in all animals. In contrast, when the AAV vector encoding huFIX was delivered into BALB/c mice using intramuscular injection, the protein was detected transiently in the serum of all animals and a strong humoral response was observed uniformly. The results indicate that the long-term systemic delivery and expression of a foreign gene can be observed following a subcutaneous injection of recombinant AAV in the presence of a functional immune system.

Recombinant AAV vector particles encoding mouse erythropoietin also were prepared and injected subcutaneously as described above in immunocompetent

BALB/c mice. The gene of interest is syngeneic with the host. Expression of mouse erythropoietin was monitored in serum samples collected over time. Over the time period monitored, expression of mouse erythropoietin was assayed by hematocrit and high level expression was observed.

Subcutaneous delivery is a very attractive mode for in vivo gene delivery because the skin is not a vital organ and is very easy to access. If a disease is caused by a defective gene product which is required to be produced and/or secreted, such as in hemophilia, diabetes and Gaucher's disease etc., subcutaneous delivery is a good candidate mode of delivering the gene product.

Actual delivery of the recombinant AAV vectors is accomplished by using any physical method that will transport the AAV recombinant vector into the subcutaneous region of a host. As used herein, "AAV vector", means both a bare recombinant AAV vector and recombinant AAV vector polynucleotides packaged into viral coat proteins, as is well known for AAV administration. Simply dissolving an AAV vector in Hanks' Balanced Saline Solution or phosphate buffered saline has been demonstrated to be sufficient to provide a vehicle useful for subcutaneous injection followed by expression distant from the site of

injection. There are no known restrictions on the carriers or other components that can be coadministered with the vector (although compositions that degrade polynucleotides should be avoided in the normal manner with vectors).

Pharmaceutical compositions can be prepared as injectable formulations or as topical formulations to be delivered subcutaneously or by transdermal transport, including implantable subcutaneous pumps (known by those of skill in the art and described in, for example, U.S. Pat. No. 5,474,552. Numerous formulations for subcutaneous injection and transdermal transport have been developed and can be used in the practice of the instant invention. The vectors can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

For purposes of subcutaneous injection, solutions in an adjuvant, such as sesame or peanut oil, or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of the AAV vector as a free acid (DNA contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a

surfactant, such as hydroxypropylcellulose. A dispersion of AAV viral particles also can be prepared in glycerol, liquid polyethylene glycols and mixtures thereof, and in oils. Under ordinary conditions of storage and use, the preparations contain a preservative to prevent the growth of microorganisms. The sterile aqueous media employed all are obtainable readily by standard techniques well known to those skilled in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that the final preparation can be administered by the device means selected.

The pharmaceutical must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils.

The proper fluidity can be maintained, for

example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like.

In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the AAV vector in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other inert ingredients from those enumerated above.

In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique which yield a powder of

the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

For purposes of topical administration, dilute sterile, aqueous solutions, usually in about 0.1% to 5%, or greater as needed, concentration, otherwise similar to the above parenteral solutions, are prepared in containers suitable for delivery by a transdermal patch, and can include known carriers, such as pharmaceutical grade dimethylsulfoxide (DMSO).

Alternatively, other known formulations for preparing topical preparations, such as ointments, creams, washes, drops, sprays and the like can be exercised as known in the art for preparing such forms for use at the dermal surface or as drops and sprays for instillation, for example in the eye or nose. In the context of the instant invention, "topical", is meant to indicate any local or particular readily accessible locale of the host, for application of the invention of interest. Thus, ointments and creams to the surface of the skin, drops or ointment for instillation, for example, in the ear or nose, sprays or mists for instillation in the nose or mouth, suppositories and the like are contemplated to fall within the scope of the instant invention.

Topical administration may be coupled with slight

abrasion of the skin to enable ready access past the keratinized epidermis to the dermis, underlying connective tissue, muscle, tissue spaces and the circulatory system.

The therapeutic compounds of the instant invention may be administered to a mammal alone or in combination with pharmaceutically acceptable carriers. As noted above, the relative proportions of active ingredient and carrier are determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice.

Also, as is well known in the art, the rAAV particles can be encapsulated in microvessels, such as liposomes, microcapsules, microbeads and the like. Standard pharmaceutic manufacturing methods can be used to make the microcapsules and microbeads. Also, standard methods for making liposomes and other such lipid membrane structures encircling an aqueous core can be made practicing methods known in the art.

The dosage of the instant therapeutic agents which will be most suitable for prophylaxis or treatment will vary with the form of administration, the particular recombinant AAV vector chosen and the physiological characteristics of the particular patient under treatment. Generally, small dosages

will be used initially and, if necessary, will be increased by small increments until the optimum effect under the circumstances is reached. Exemplary dosages for humans includes within the range of 10^8 up to approximately 5 x 10^{14} particles in a total volume of up to about 3-10 ml, subcutaneously injected in aliquots into different sites on the human body, including the thighs, arms and back.

Since AAV has in the past been shown to have a broad host range and now has been demonstrated to be operable via subcutaneous injection, there are no known limits on the hosts in which the herein described methods of delivery can take place, particularly in mammals, birds, fish and reptiles, especially domesticated mammals and birds, such as cattle, sheep, pigs, horses, dogs, cats chickens and turkeys. Both human and veterinary uses are preferred.

The gene being expressed can be either a DNA segment encoding a protein, with whatever control elements (e.g., promoters, operators) are desired by the user, or a non-coding DNA segment, the transcription of which produces all or part of some RNA-containing molecule, such as a ribozyme or an anti-sense molecule. Since the spirit of the instant invention is directed to a route of delivery and to

the vector rather than to the material being delivered, there are no limitations on the foreign DNA (non-AAV DNA) being delivered by the vector. The gene need not be limited to those strictly useful in the subcutaneous or topical region of administration since the host vascular system will deliver the gene product to other parts of the body. Also, the AAV particles themselves may enter the circulatory system and be transported to sites distal from the point of administration.

Different vectors, such as naked DNA, adenovirus and retrovirus, have been utilized to deliver various transgenes into various tissues, including muscle. However, such systems cannot offer both high efficiency and long term expression. For naked plasmid DNA directly delivered into muscle tissue, the efficiency is low. There are only a few cells near the injection site that can maintain transgene expression. Furthermore, the plasmid DNA in the cells remains as non-replicating episomes, i.e. in the unintegrated form. Therefore, eventually it will be lost.

Adenovirus vectors can infect the non-dividing cells and therefore, can be delivered directly into the mature tissues, such as muscle. However, the transgenes delivered by adenovirus vectors are not

useful to maintain long term expression. Since adenovirus vectors retain most of the viral genes, those vectors pose potential problems, i.e. safety. The expression of those genes can cause the immune system to destroy the cells containing the vectors (see, for example, Yang et al. 1994, Proc. Natl Acad. Sci. 91:4407-4411). Thus, an adenovirus vector cannot be delivered repeatedly. Also, because adenovirus is not an integration virus, the DNA eventually will be diluted or degraded in the cells.

Although retrovirus vectors achieve stable integration into the host chromosomes, the use thereof is restricted because currently-used vectors only infect dividing cells whereas a large majority of target cells are non-dividing.

Adeno-associated virus vectors have certain advantages over the above-mentioned vector systems. First, like adenovirus, AAV efficiently can infect non-dividing cells. Second, all the AAV viral genes are eliminated in the vector. Since the viral gene expression-induced immune reaction is no longer a concern, AAV vectors are safer than adenovirus vectors. Third, AAV is an integration virus by nature and integration into the host chromosome will maintain the transgene in the cells. Fourth, AAV is an extremely stable virus which is resistant to many

detergents, pH changes and heat (stable at 56°C for more than an hour). AAV also can be lyophilized and redissolved without losing significant activity. Finally, AAV causes no known diseases or pathogenic symptoms in humans. Therefore, it is a very promising delivery vehicle for gene therapy.

Two recent review articles provide a particularly complete overview of the recent status of gene therapy using AAV vectors and include a collection of additional recent scientific publications in this field: Samulski, R. J., "Adeno-associated Viral Vectors," Chapter 3 in "Viruses in Human Gene Therapy", Chapman & Hall, J.-M. H. Vos., ed. (1994) and Samulski, R. J., "Adeno-associated Virus-based Vectors for Human Gene Therapy", Chapter 11 in "Gene Therapy: From Laboratory to the Clinic", World Scientific, K. M. Hui, ed. (1994).

The instant invention now will be exemplified in the following non-limiting examples.

EXAMPLES

EXAMPLE 1. RECOMBINANT AAV VECTOR: rAAV-MFG-HUMAN-FACTOR IX.

The recombinant AAV vector for expressing human Factor IX contains an MFG promoter, which is derived

from the murine Moloney virus long terminal repeat, the entire human Factor IX cDNA and bGH pA, which is the bovine growth hormone polyadenylation site, see FIG. 1. Briefly, SSV9-MFG-ShF9 (huFIX) (FIG. 23) was generated by cloning a 3.24 kbp NheI-BamHI fragment containing the Moloney murine leukemia virus (MLV) 5′ LTR, adjacent splice donor/acceptor sequence and huFIX cDNA sequence precisely connected to the MLV env ATG from plasmid MFG-S-huFIX into XbaI digested SSV9. The MLV 3′ LTR of the MFG-S-huFIX vector was replaced with the poly(A) site of bovine growth hormone from pRc/CM (Invitrogen). For a description of MFG-S see Dranoff et al. (1993) Proc. Natl. Acad. Sci. 90:3539-3543. The huFIX cDNA in MFG-S-huFIX (FIG. 24) was obtained from pAFFIXSVNeo, see St. Louis & Verma (1988) PNAS 85:3150-3154.

EXAMPLE 2. RECOMBINANT AAV VECTOR: rAAV-MD-HUMAN INTERFERON.

The recombinant AAV vector for expressing human interferon (FIG. 2) contains an MD promoter, which is derived from the Cytomegalovirus immediate early promoter, the entire human interferon cDNA and a β-globin polyadenylation site.

Plasmid SSV9-MD AAV (pSSV9/MD-2) (FIG. 20) was constructed by subcloning a 2.35 kbp XbaI fragment

from pMDG (Naldini et al. (1996) Science 272:263-267). pSSV922 MD-2 contains the Cytomegalovirus (CMV) immediate early gene promoter/enhancer (nucleotide positions 195-993), exon 2 and 3, intervening sequence 2 (IVS2) (nucleotide position 1036-1561) and a polyadenylation signal (nucleotide positions 1573-2355) derived from the human β-globin gene. The fragment was modified by PCR to contain restriction sites for PmlI, EcoRI and BglII at nucleotide positions 1562, 1567, and 1573, respectively, for subcloning of selected transgenes.

pSSV9-MD-hu alpha interferon (huAIFN) (FIG. 22) was constructed by insertion of the huIFN sequence between the PmlI and BglII cloning sites of pSSV9-MD2 (FIG. 20). The huIFN cDNA also was generated by PCR using multiple overlapping oligonucleotides (FIG. 22).

EXAMPLE 3. RECOMBINANT AAV VECTOR: rAAV-MD-mouse EPO.

The recombinant AAV vector for expressing mouse EPO contains an MD promoter, derived from the cytomegalovirus immediate early promoter, the entire mouse erythropoietin cDNA and a β-globin polyadenylation site (FIG. 3).

pSSV9-MD2-murine erythropoietin (muEPO) (FIG. 21) was constructed by insertion of the muEPO cDNA between the PmlI and BglII cloning sites of pSSV9-MD-2

(FIG. 20). In that construct, the muEPO gene is flanked by the CMV promoter, β-globin intron and the β-globin poly(A). The 579 base pair muEPO coding region was generated by PCR using multiple overlapping oligonucleotides (Dillion & Rosen (1990) Biotechniques 9:298-299) and contained compatible PmlI and BglII sites for subcloning into the vector backbone.

EXAMPLE 4. RECOMBINANT AAV VECTOR: rAAV-CMV-LACZ.

The rAAV-CMV-LacZ construct, see FIG. 4, is known as pdx-31-LacZ and is described in McCown et al. (1996) Brain Res. 713:99-107.

EXAMPLE 5. PACKAGING OF THE RECOMBINANT AAV VECTORS.

The recombinant AAV vectors were packaged as described by R. Snyder et al., "Production of recombinant adeno-associated viral vectors," in Current Protocols in Human Genetics, pp. 12.1.1-12.1.24, N. Dracopoli et al., eds. (John Wiley & Sons, New York, 1996).

EXAMPLE 6. FACTOR IX EXPRESSION IN IMMUNOCOMPETENT MICE.

Recombinant AAV-MFG-Human Factor IX was administered subcutaneously as a single injection of either 5.9×10^{10}, 5.9×10^{9} or 5.9×10^{8} particles in

a volume of 300 microliters of Hanks' Balanced Salt Solution (HBSS) on the dorsal side of BALB/c mice (each dose of recombinant AAV vector was administered to a group of five mice). Mice were bled using heparin-coated capillary tubes. Blood was collected into EDTA-coated tubes at the indicated number of days after subcutaneous injection depicted in FIGS. 5, 6 and 7, and stored frozen. The expression of human Factor IX was determined by ELISA using monoclonal or polyclonal antibodies for human Factor IX (monoclonal anti-factor IX antibody from Boehringer Mannheim, Cat. # 1199 277, rabbit anti-human factor IX from DAKO, Cat. # A300 and Asserachrom® IX:Ag test kit, Cat. # 00564). Individual animals expressing human Factor IX at detectable levels are identified by number: 100, 108, 202, 204, 208 and 306.

EXAMPLE 7. LEVELS OF ANTI-HUMAN FACTOR IX AND ANTI-AAV ANTIBODIES CORRELATE WITH GENE EXPRESSION.

Animals from FIG. 5, 6 and 7 were tested for the presence of antibodies against human Factor IX and AAV, see FIGS. 8A and 8B. Animal numbers correspond to the numbers indicated in FIG. 5, 6 and 7. Animal 400 is a control animal injected with HBSS.

The presence of antibodies to the AAV capsid proteins was determined by an ELISA as follows:

1×10^{10} functional rAAV-LacZ purified virions ($\sim 5 \times 10^{12}$ total particles) in 100 μl were used to coat the wells of 96-well plates for 16 hours. The unbound virions were washed from the plate, the plate was blocked and incubated with 200 μl of 1:50, 1:800 and 1:600 dilutions of mouse serum for 2 hours. The plate was developed using an anti-mouse horseradish peroxidase-conjugated antibody and 1 mg/ml O-phenylenediamine dihydrochloride substrate (Sigma) in 7.5% H_2O_2. Titers were calculated by determining the serum dilution at which the OD value was half-maximal.

Titers of antibody of hFIX were determined by an ELISA on selected days postinjection. Animals that expressed detectable levels of hFIX for the duration of the experiment are indicated by the plus (+) sign.

EXAMPLE 8. COMPARISON OF LONG-TERM GENE EXPRESSION IN IMMUNOCOMPETENT AND IMMUNOCOMPROMISED MICE.

rAAV-MFG-human Factor IX was administered subcutaneously once as an injection of either 5.9×10^{10}, 5.9×10^{9} or 5.9×10^{8} particles in a volume of 300 microliters of HBSS on the dorsal side of BALB/c or NIH-3 mice (each dose of vector was administered to a group of five mice). Mice were bled using heparin-coated capillary tubes. The blood was

collected into EDTA-coated tubes at the time points indicated in FIGS. 9A and 9B and stored frozen. The expression of human Factor IX was determined by ELISA, and the results are shown in FIGS. 9A and 9B. Individual BALB/c animals expressing human Factor IX at detectable levels are identified by number: 103, 104 and 204. The expression of human Factor IX for immunocompetent mice correlated positively to the amount of recombinant AAV vector administered.

EXAMPLE 9. THE LEVELS OF ANTI-HUMAN FACTOR IX CORRELATE WITH GENE EXPRESSION.

Animals from FIG. 9A were tested for the presence of antibodies against human Factor IX. Antibody titers were determined by ELISA and the results are depicted in FIG. 10.

EXAMPLE 10. LONG-TERM EXPRESSION OF HUMAN INTERFERON IN IMMUNOCOMPETENT AND IMMUNOCOMPROMISED MICE.

rAAV-MD-human interferon was administered subcutaneously once as a single injection of either 8.5×10^{10}, 8.5×10^{9} or 8.5×10^{8} particles in a volume of 300 microliters of HBSS on the dorsal side of BALB/c or NIH-3 mice (each dose of vector was administered to a group of five mice). Mice were bled using heparin-coated capillary tubes. Blood was

collected into EDTA-coated tubes at the time points indicated in FIGS. 11A and 11B and stored frozen. The expression of human interferon was determined by ELISA.

EXAMPLE 11. EXPRESSION OF MOUSE ERYTHROPOIETIN IN IMMUNOCOMPETENT BALB/c MICE.

rAAV-MD-mouse-erythropoietin was administered subcutaneously once as a single injection of either 8.9×10^{10} or 8.9×10^{9} particles in a volume of 100 microliters of HBSS on the dorsal side of BALB/c mice (each dose of vector was administered to a group of six or eight mice). Mice were bled at the time points indicated on FIG. 12 and the hematocrit was determined.

EXAMPLE 12. EPO EXPRESSION.

Epo protein levels were determined at day 105 for mice in FIG. 12. Epo protein levels were determined by radioimmunoassay (RIA), see FIG. 13.

EXAMPLE 13. COMPARISON BETWEEN INTRAMUSCULAR AND SUBCUTANEOUS EXPRESSION OF MOUSE ERYTHROPOIETIN IN IMMUNOCOMPETENT MICE.

rAAV-MD-mouse erythropoietin was administered subcutaneously once as a single injection of either

8.9×10^{10}, 8.9×10^{9} or 8.9×10^{8} particles in a volume of 100 microliters of HBSS on the dorsal side of BALB/c mice or **intramuscularly** as a single injection of either 8.9×10^{10}, 8.9×10^{9} or 8.9×10^{8} particles in a volume of 30 microliters of HBSS to the quadriceps of BALB/c mice (each dose of vector was administered to a group of three mice). Mice were bled at the time points indicated in FIGS. 14A and 14B and the hematocrit was measured.

EXAMPLE 14. EPO EXPRESSION.

Epo protein levels were determined at day 180 for mice in FIGS. 14A and 14B. Epo protein levels were determined by RIA, see FIG. 15.

EXAMPLE 15. EXPRESSION OF HUMAN INTERFERON ADMINISTERED INTRAVENOUSLY IN IMMUNOCOMPETENT MICE.

rAAV-MD-human interferon (8.5×10^{10} particles) was administered intravenously as a single injection in a volume of 100 microliters of HBSS into the tail vein of BALB/c (the dose of vector was administered to a group of five mice). Mice were bled using heparin-coated capillary tubes. Blood was collected into EDTA-coated tubes at the indicated times and stored frozen. The expression of human interferon was determined by ELISA (Endogen) and the results are

shown in FIG. 16.

EXAMPLE 16. EXPRESSION OF HUMAN FACTOR IX ADMINISTERED TO THE MUSCLE IN IMMUNOCOMPETENT MICE.

rAAV-MFG-human Factor IX (2.5×10^{10} particles) was administered intramuscularly as a single injection in a volume of 50 microliters of Hanks' Balanced Salt Solution (HBSS) into the quadriceps of BALB/c mice (the dose of vector was administered to a group of five mice). Mice were bled using heparin-coated capillary tubes. The blood was collected into EDTA-coated tubes at the indicated times and stored frozen. The expression of human Factor IX was determined by ELISA and the results are shown in FIG. 17.

EXAMPLE 17. LOCATION OF rAAV-CMV-LACZ EXPRESSION.

Recombinant AAV-CMV-LacZ (6.9×10^7 functional particles) was administered subcutaneously as a single injection in a volume of 300 μl PBS on the dorsal side of four BALB/c mice. Two weeks and six weeks after injection, two mice from the rAAV-CMV-LacZ group and one mouse from the PBS control group were euthanized and skin samples, muscle samples, inguinal lymph nodes, inguinal fat pads and spleen were harvested for histology. The skin samples were obtained by raising

the skin at the injection site with forceps, forming a tent as described for injection and cutting around the base of the tent of skin. The muscle samples were collected just lateral on both sides of the dorsal midline in the lumbar region. Inguinal lymph nodes and fat pads were collected from both the left and right side of each mouse.

To detect ß-galactosidase protein, tissue samples were flash frozen, cut into 10 μm sections and collected onto glass slides. The sections were fixed with 0.5% glutaraldehyde for 10 min., washed with PBS and stained with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) using standard procedures. The sections then were washed with PBS and counterstained with nuclear fast red.

ß-galactosidase protein activity was detected in the skin tissue, such as, in the panniculus carnosus, a skeletal muscle layer located in the skin, see FIG. 19. ß-galactosidase activity was not present in non-injected animals, see FIG. 18.

All references cited in the specification are herein incorporated by reference in entirety to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WE CLAIM:

1. A method of expressing a gene product in an animal, comprising:

administering subcutaneously or topically a recombinant adeno-associated virus (AAV) vector to an animal, wherein said vector comprises a non-AAV gene of interest ligated into an AAV vector.

2. The method of Claim 1, wherein said administering step occurs either once or repeatedly at intervals.

3. The method of Claim 1, wherein said vector is administered dissolved or suspended in a pharmaceutically acceptable carrier.

4. The method of Claim 3, wherein said liquid carrier comprises an aqueous solution.

5. The method of Claim 1, wherein said gene comprises a DNA segment encoding a therapeutic gene product operably linked to a promoter such that expression of said polypeptide occurs after said recombinant AAV vector is administered.

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6. The method of Claim 5, wherein said expressed gene product is diffusible from the site of administration.

7. The method of Claim 1, wherein said recombinant AAV vector comprises a non-AAV gene of interest ranging in length from 1,000 to 5,300 nucleotides in length, ligated into an AAV vector.

8. The method of Claim 1, wherein said gene comprises a polynucleotide encoding an anti-sense RNA molecule or a genetic control element.

9. The method of Claim 1, wherein said animal is a mammal.

10. The method of Claim 1, wherein said animal is a human.

11. The method of Claim 1, wherein said non-AAV gene of interest comprises a polynucleotide encoding interferon.

12. The method of Claim 1, wherein said non-AAV gene of interest comprises a polynucleotide encoding Factor IX.



13. The method of Claim 1, wherein said non-AAV gene of interest comprises a polynucleotide encoding erythropoietin.

14. The method of Claim 1, wherein said administering step is subcutaneous administration.

15. The method of Claim 14, wherein said administering subcutaneously comprises using an implantable pump.

16. A method of gene therapy, comprising: administering to an animal subcutaneously or topically a recombinant adeno-associated virus (AAV) vector, wherein said vector comprises a non-AAV gene of interest ligated into an AAV vector, and wherein an expression product of said recombinant AAV vector is diffusible from a site of administration.

17. A recombinant adeno-associated virus (AAV) vector, comprising:

a non-AAV gene of interest, wherein said gene of interest encodes a diffusible polypeptide,

a promoter and a polyadenylation sequence,

wherein said recombinant AAV vector is suitable

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for subcutaneous injection and results in expression of said diffusible polypeptide.

18. A composition comprising a recombinant adeno-associated virus (AAV) vector comprising a non-AAV gene and regulatory elements for expressing same, and a pharmaceutically acceptable carrier, diluent or excipient suitable for subcutaneous or topical administration.

19. The composition of Claim 18, further comprising dimethylsulfoxide.

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