BJCP British Journal of Clinical Pharmacology

DOI:10.1111/bcp.12010

Gene doping: gene delivery for olympic victory

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Keywords
detection, gene doping, gene therapy, secreted proteins, sport, transcription factors

Received
30 July 2012

Accepted
3 October 2012

Accepted Article Published Online
22 October 2012

With one recently recommended gene therapy in Europe and a number of other gene therapy treatments now proving effective in clinical trials it is feasible that the same technologies will soon be adopted in the world of sport by unscrupulous athletes and their trainers in so called 'gene doping'. In this article an overview of the successful gene therapy clinical trials is provided and the potential targets for gene doping are highlighted. Depending on whether a doping gene product is secreted from the engineered cells or is retained locally to, or inside engineered cells will, to some extent, determine the likelihood of detection. It is clear that effective gene delivery technologies now exist and it is important that detection and prevention plans are in place.

Introduction

Gene therapy generally involves the delivery of genetic material encoding the expression of proteins that are either endogenous or biological for treatment of disease. Gene therapy has great potential to treat genetic diseases and holds much promise as a mode of delivering biological molecules in more widespread conditions. After initial hype and several lows the first gene therapy product has now been recommended for approval in Europe [1] and there have been a number of recent clinical trials that have demonstrated the effectiveness of this treatment modality.

All gene therapy approaches use a vector to deliver the genetic material to cells and also utilize the transcriptional machinery of the cell for gene expression. The delivery vector can be as simple as naked plasmid DNA or as complex as replication deficient recombinant viruses that have innate ability to deliver genes. The first gene therapy successes were achieved in X-linked severe combined immunodeficiency (SCID) patients through *ex vivo* engineering their bone marrow stem cells (BMSC) with retrovirus encoding a correct copy of the common cytokine-receptor gamma chain prior to re-implantation [2, 3]. Although only a small percentage of cells are corrected these have a selective advantage and are able to populate the patient with a functioning immune system. This approach of engineering BMSC has been similarly adopted in the successful treatment of other genetic conditions including adenosine deaminase (ADA) SCID [4] and X-linked chronic granulomatous disease [5].

Patients with Leber's congenital amaurosis have a retinal degeneration with severe vision loss noted in early infancy that leads to blindness. In one form of the disease there are mutations in retinal pigment epithelium-specific protein 65 kDa (RPE65). In these patients adeno-associated virus (AAV) 2 has been used as a vector to deliver a correct copy of the RPE65 gene to retinal cells following sub-retinal injection and the genetically corrected cells remain local to the gene delivery site [6, 7]. Treated patients had an improved field of vision and their ability to negotiate an obstacle course also improved. Another example of local gene therapy has been in Parkinson's disease with targeted intracerebral delivery of glutamic acid decarboxylase (GAD) into the subthalamic nucleus using an AAV2 vector [8] which demonstrated improved motor score after 6 months compared with controls in a double-blind randomized clinical trial [9]. The most recent advance in the field of gene therapy has been the successful delivery of a

factor IX gene in patients with haemophilia B [10]. This was achieved by delivering an AAV-8 intravenously which has a tropism for the liver. The transduced hepatocytes in the liver then secrete the factor IX. So in these patients the engineered cells localized in the liver secrete a protein that is distributed systemically.

With the increasing number of gene therapy successes there is an increasing armoury in the gene therapist's toolbox which in time will facilitate the application of gene therapy in other diseases. Another arena where gene therapy techniques could be utilized for non-therapeutic purposes is 'gene doping', which is defined by the World Anti-Doping Agency (WADA) as 'the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance'. Gene therapy approaches deliver genetic material to the body's own cells which then produce the encoded protein. In this way the expressed protein can potentially be indistinguishable from the endogenous version of the same protein encoded from chromosomal genes. This similarity is important for treatment of disease to ensure that the expressed protein is not targeted by the immune system so that long term production of the gene therapy protein is achieved. This ability to produce biologically active molecules that are potentially identical to endogenous proteins is also appealing for unscrupulous athletes as a way to deliver undetectable performance enhancing molecules.

In general, doping strategies aim to improve metabolic activity or enhance tissue function. There are some proteins including erythropoietin (EPO) and growth factor [11] that have been used as doping agents and these will be likely candidates for delivery in genetic form.

Gene doping targets

Oxygen delivery

The variables that control oxygen delivery include erythrocyte number in the blood, degree of tissue vascularization and rate of blood flow. Oxygen is delivered to the body through breathing and so improving lung function is a potential target for gene doping. Gene delivery to the lungs has primarily been developed for the treatment of cystic fibrosis, a genetic condition caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR) which regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs. Gene therapy for the condition requires delivery of the correct CFTR gene to the lungs of patients for expression in the epithelium. Expressing genes that improve oxygen delivery could be achieved in lower airways if gaseous exchange could be improved. Alternatively targeting pulmonary muscle could facilitate improved lung function.

Erythropoietin (EPO) is a glycoprotein produced predominantly by the kidney, which acts on erythoid progenitor cells in the bone marrow to regulate red blood cell production by the process of erythropoiesis which increases haemoglobin and haematocrit and therefore increases oxygen delivery. This type of systemic delivery of a protein can potentially be achieved by gene therapy. Gene delivery of EPO has been demonstrated in several experimental studies and using a variety of vectors including intramuscular injection of EPO encoding AAV in non-human primates [12].

Because gene therapy can also be used to have local effects at the site of gene delivery it is also possible to produce proteins locally whose effects are restricted to the injection site for example the induction of the growth of new blood vessels by the process of angiogenesis. Increased blood supply will enhance oxygen delivery which could be significant in many clinical settings such as peripheral vascular disease (PVD), coronary artery disease (CAD) and wound healing. There has been progress in the development of gene therapy for the induction of angiogenesis for the treatment of CAD and PVD. Several clinical trials have examined expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) from vectors including plasmid DNA and adenovirus. Whist there are reports of safety with these approaches there are no clear cut demonstrations of therapeutic effect in well controlled clinical trials [13].

Glucose metabolism

Genetic delivery would also offer new doping approaches such as the local expression of proteins within a target organ. The liver plays an essential role in both glucose storage (glycogenesis) and in the generation of glucose (gluconeogenesis) from non-carbohydrate carbon substrates including lactate which is converted into pyruvate by lactate dehydrogenase through the Cori cycle. Through local gene delivery to the liver it may be possible to enhance either storage or liberation of glucose in the liver. There have been no gene therapy studies that have aimed to alter glucose metabolism directly in healthy subjects but clearly treatment of diabetic patients is a potential application of gene therapy. One study in diabetic obese mice has shown that continuous expression of glucagon-like peptide (GLP)-1 from an adenoviral vector administered by intravenous injection resulted in long term remission of diabetes by improving insulin sensitivity through restoration of insulin signalling and reduction of hepatic gluconeogenesis [14].

Muscle growth

The liver is also the organ that secretes most insulin-like growth factor-1 (IGF-1) which is stimulated by growth hormone. It has several effects on skeletal tissues including skeletal muscle hypertrophy and blocking muscle atrophy and it is also protective for cartilage cells. Interestingly,

when the IGF-1 gene was combined with regions from the avian skeletal α-actin gene, expression of IGF-1 in a transgenic mouse resulted in muscle hypertrophy and increase in expressed IGF only occurred within the muscle and with no increase in systemic concentrations of IGF-1 [15]. The potential for increasing muscle mass and strength with IGF-1 and the knock on influence of training was examined by Lee *et al*. [16]. In this study IGF-1 over-expression following AAV vector delivery increased muscle size in untrained rats and a further increase in muscle mass was observed when rats were also resistance trained (ladder climbing). Furthermore when rats were detrained there was slower loss of muscle mass in rats that were treated with the IGF-1 vector.

Myostatin is an interesting protein as it is an inhibitor of muscle growth. In a recent case a boy with inactive myostatin displayed hypertrophic limb muscle development, and at 4.5 years was able to hold two 3 kg dumbbells in horizontal suspension with extended arms [17]. Recent work in racehorses has also linked different polymorphisms of the gene to suitability to sprint or stamina races [18]. By preventing the action of myostatin it may be possible to increase muscle growth and one way to achieve this could be with another endogenous protein called follistatin. Myostatin binds to activin type II receptors (Act RII), particularly Act RIIB, and this interaction can be inhibited by follistatin, an activin-binding protein [19]. When follistatin was expressed in quadriceps of non-human primates following delivery with an AAV1 encoding vector an increase in muscle size of 15% was observed while strength, measured in twitch force was increased by 26.3% and these changes persisted for the 6 month period of the experiment [20].

Preventing pain

Conquering pain is essential to push your body to new levels of performance. The potential to reduce the perception of pain, during exertion may allow athletes to push for longer and perform better. Gene therapy for the treatment of pain is currently being examined. Research has utilized replication deficient herpes simplex virus (HSV) encoding preproenkephalin delivered into the paws of mice. The HSV is neurotropic, able to enter nerve endings at the injection site and travel retrogradely to neuronal cell bodies in the dorsal root ganglia where transgene is expressed into preproenkephalin which reduces the response to noxious stimuli [21]. The preproenkephalin is a precursor for enkephalin production which acts on opioid receptors as a naturally occurring analgesic. A phase 1 trial, using this method, in patients with intractable pain caused by cancer [22] has shown safety of the approach but further study is necessary to determine its effectiveness. Although gene therapy holds promise for those suffering with chronic pain, the pain experienced by athletes is often more intermittent and is confined to periods of exertion. If pain relief is to become a target for gene doping, the therapy would have to desensitize the patient to pain during these times of high intensity, not just at a constant baseline level.

Gene doping with intracellular molecules

Gene therapy requires genetic material to be delivered to the cell nucleus for the therapeutic genes to be expressed by the transcriptional machinery of the cell. Depending on the encoded gene product it will either be secreted from the cell or remain intracellular. It is this potential to deliver a gene whose products remain inside the engineered cells that provides opportunities for gene doping that will be extremely difficult to detect and is not feasible with delivery of recombinant proteins which act outside the cell. Candidate genes that could encode intracellular proteins include transcription factors and activators, enzymes or RNA molecules.

Transcription factors

Type 1 muscle fibres have many mitochondria and are richly supplied by blood capillaries which allow large amounts of ATP to be produced under oxidative metabolism which means that these fibres are fatigue resistant. Type II fibres have lower levels of mitochondria and use glycolytic metabolism to produce ATP and are therefore more susceptible to fatigue. The fast manner in which the ATP is produced in type II fibres results in quick, more powerful contractions which are more important in sports such as sprinting. It is well known that endurance exercise induces phenotypic changes in skeletal muscles that lead to enhanced exercise capacity and improved metabolic homeostasis [23]. More recently peroxisome proliferator-activated receptors (PPARs), which are nuclear receptor proteins that function as transcription factors, have been shown to have a role in regulating the fibre type within a muscle. Generation of transgenic mice in which an activated form of peroxisome proliferator-activated receptor delta (PPARδ) was expressed in skeletal muscle, induced an increased number of type I muscle fibres [24]. It was noted that these mice had an increased numbers of type I fibres compared with controls, both in normal type I rich muscle, such as soleus, and mixed type II muscle, such as gastrocnemius. These mice could also run further and for longer compared with controls, suggesting that the type I fibres generated from PPAR-δ gene expression produced a beneficial effect on the mice by increasing physical performance. Similarly, peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) is a transcriptional co-activator which, when over expressed in muscle, promotes a fibre switch towards oxidative type I slow fibres. Recently is has been shown that this effect is mediated via the transcription factor hypoxia-inducible factor (HIF) 2α [25]. The lack of oxygen in muscle during vigorous exercise causing hypoxia is also known to induce HIF-1α, a transcription factor that induces expression of target genes including the angiogenic factor VEGF and

EPO [26]. These HIF transcription factors are degraded by prolyl hydroxylase enzymes under normoxic conditions but these enzymes are inactive in hypoxia permiting HIF activity. It is however possible to engineer a stable version of HIF-1α by point mutation of the proline residues that are subject to prolyl hydroxylation. In the mutated form HIF-1α is constitutively active [27] and can be delivered to muscle genetically to enhance vascularization [28] and potentially promote erythropoesis. In theory, synthetic transcription factors could also be generated that have similar effects such as that engineered by Dai *et al*. using zinc finger domains targeted to the VEGF locus which when expressed *in vivo* in mice induced angiogenesis [29].

Enzymes

An example of an intracellular enzyme that could be used in gene doping to reduce pain is GAD which controls production of the inhibitory neurotransmitter GABA from glutamate, which has an analgesic effect. Expression of GAD from an HSV vector into the dorsal root ganglia of rats with T13 hemisections showed a reduction in pain perception below the level of the lesion [30].

Small inhibitory RNA (siRNA)

Endogenous gene expression can be regulated at the level of micro RNA (miRNA) molecules which target messenger RNA (mRNA) molecules. Synthetic siRNA molecules utilize the endogenous machinery of the cell to inhibit protein translation in a similar manner to miRNA. In addition siRNA molecules can also be produced long-term inside cells as short hairpin (sh) RNA molecules that are processed inside the cell into siRNA. Both these types of RNA molecule could be utilized in gene doping strategies where inhibition of a protein's synthesis could enhance performance. An obvious target is myostatin and studies using either siRNA or shRNA targeting myostatin mRNA have demonstrated an increase in muscle size and mass [31, 32].

Detection of gene doping

With the continued development of gene therapy the potential for gene doping increases (Figure 1). Detection of gene doping is difficult because expressed proteins can be identical to endogenous versions of the same protein. Where gene encoded proteins are secreted from cells it maybe possible to at least detect them in biological samples and in some cases it may be possible to differentiate between the endogenous molecule and 'doping' protein by differences in glycosylation as previously demonstrated with recombinant and endogenous EPO [33]. If doping genes express proteins or RNA molecules that remain inside the engineered cell or are retained at their site of production, then detection will only be possible by directly sampling tissue from the doping site. Without knowledge of a precise gene doping site then multiple samples (biopsies) will be required for detection which will not be tolerated by athletes and authorities. There are other possibilities for detection of gene doping such a immune response to the delivery vector or monitoring the metabolic profile in biological samples but these will only provide an indirect indication of gene doping and not conclusive proof. Detection of gene doping could be further complicated if the doping gene is expressed from a regulated expression system which could mean that expression can be switched 'on' and 'off'. The way in which the switch is controlled could be through use of another chemical such as an antibiotic [34], a combination of chemical regulation and endogenous demand [35] or in response to the demands of training and performance alone. An example of this is Repoxygen™ developed by Oxford Biomedica in 2002 which comprises of a viral-vector with a human EPO gene expressed from a promoter that is activated by HIF transcription factors and therefore production of EPO is self-regulated, only being induced (when required) under hypoxic conditions. This product delivered by intramuscular (i.m.) injection has been assessed in experimental models for the treatment of anaemia. Gene doping with self-regulated genes of this sort will be even more difficult to detect.

Gene doping risks

The development of gene therapy has not been a smooth journey to date. There have been some major setbacks along the way which have usually been a consequence of the vector used to deliver the therapeutic gene. Pre-existing immunogenicity in patients is caused by prior exposure to infectious forms of the virus and this can be a fundamental problem which limits effectiveness of gene delivery and results in immune killing of transduced cells [36]. High doses of adenovirus delivered to Jesse Gelsinger caused a massive inflammatory response with an 'immune revolt' where the immune system targeted vital organs leading to his death [37]. An area that has scarcely been explored in gene therapy trials is the re-administration of vectors which may well be attractive in gene doping but is likely to pose additional danger. Retroviruses deliver genes permanently into the genome and this can also have severe consequences. The treatment of X-linked SCID patients through *ex vivo* modification of BMSCs was initially effective but a number of children later developed T cell leukemia as a consequence of gene insertion activating the expression of oncogenes including *LMO2* [38].

The risks associated with gene doping are substantial. Gene doping is unlikely to be performed under close medical supervision/monitoring and short term gain may well be achieved at the expense of longer term health problems for athletes. It is of paramount importance that athletes and coaches are educated about the potential risks of gene doping before we read tragic headlines involving sporting heroes.

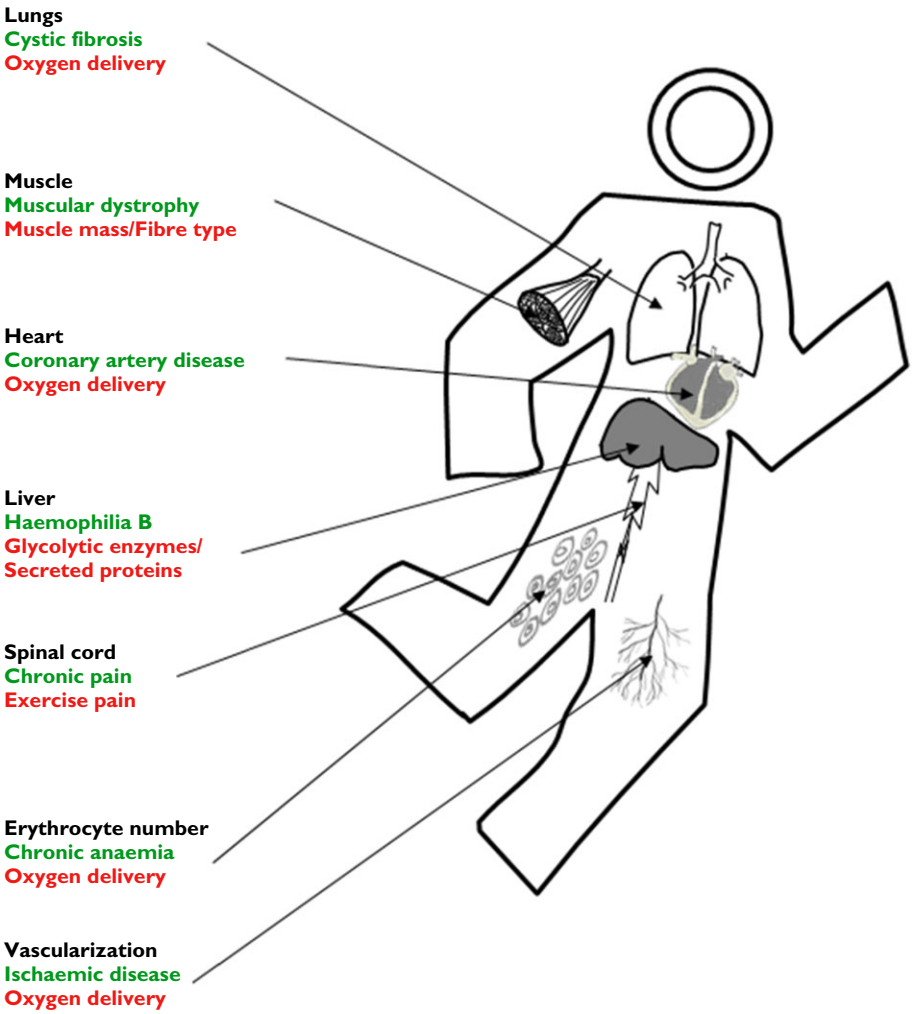

Figure 1

How and where gene doping might be done. At each site in the body a gene therapy approach is indicated in green whilst the potential for gene doping is indicated in red

Competing Interests

The author has completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf and declares no support from any organization for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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