Sports Med (2013) 43:965–977
DOI 10.1007/s40279-013-0075-4

REVIEW ARTICLE

From Gene Engineering to Gene Modulation and Manipulation: Can We Prevent or Detect Gene Doping in Sports?

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Published online: 6 July 2013

Abstract During the last 2 decades, progress in deciphering the human gene map as well as the discovery of specific defective genes encoding particular proteins in some serious human diseases have resulted in attempts to treat sick patients with gene therapy. There has been considerable focus on human recombinant proteins which were gene-engineered and produced in vitro (insulin, growth hormone, insulin-like growth factor-1, erythropoietin). Unfortunately, these substances and methods also became improper tools for unscrupulous athletes. Biomedical research has focused on the possible direct insertion of gene material into the body, in order to replace some defective genes in vivo and/or to promote long-lasting endogenous synthesis of deficient proteins. Theoretically, diabetes, anaemia, muscular dystrophies, immune deficiency, cardiovascular diseases and numerous other illnesses could benefit from such innovative biomedical research, though much work remains to be done. Considering recent findings linking specific genotypes and physical performance, it is tempting to submit the young athletic population to genetic screening or, alternatively, to artificial gene expression modulation. Much research is already being conducted in order to achieve a safe transfer of genetic material to humans. This is of critical importance since uncontrolled production of the specifically coded protein, with serious secondary adverse effects (polycythaemia, acute cardiovascular problems, cancer, etc.), could occur. Other unpredictable reactions (immunogenicity of vectors or DNA-vector complex, autoimmune anaemia, production of wild genetic material) also remain possible at the individual level. Some new substances (myostatin blockers or anti-myostatin antibodies), although not gene material, might represent a useful and well-tolerated treatment to prevent progression of muscular dystrophies. Similarly, other molecules, in the roles of gene or metabolic activators [5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), GW1516], might concomitantly improve endurance exercise capacity in ischaemic conditions but also in normal conditions. Undoubtedly, some athletes will attempt to take advantage of these new molecules to increase strength or endurance. Antidoping laboratories are improving detection methods. These are based both on direct identification of new substances or their metabolites and on indirect evaluation of changes in gene, protein or metabolite patterns (genomics, proteomics or metabolomics).

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1 Introduction

Gene therapy is acquiring considerable importance in the prospective treatment of many genetic or acquired diseases [1]. Deciphering the complete human genome enables scientists to discover the origin of some genetic diseases

and at the same time to study possible methods for treatment. In the therapeutic field, the possibility occurs of gene treatment by direct introduction of genetic material into the body or by up-regulation (or down-regulation) of the activity of some deficient (or harmful) genes. This could permit a nearly physiological and continuous production of some proteins, avoiding the periodic administration of external recombinant proteins.

Unfortunately, the same methods could be used by athletes in order to cheat by aiming to enhance the endogenous production of some particular proteins artificially [2]. Such misused gene therapies would be likely to show the same effectiveness as the actual doping methods based on the administration of exogenous recombinant molecules.

2 Evolution of the Antidoping Rules

In June 2001, for the first time, the Gene Therapy Working Group, appointed by the International Olympic Committee (IOC) Medical Commission, faced the possibility of potential gene doping abuse by athletes in a meeting on "Gene therapy and its impact on sport" [3].

In March 2002, the World Anti-Doping Agency (WADA), together with sport scientists, genetics experts and sport representatives organized a workshop in the Banbury Centre in New York to discuss the possibility of gene transfer in sport where talent and genetic manipulation collide, and later (2004) appointed an Expert Group on gene doping [4].

Introduced first in 2003 in the IOC/WADA list, gene doping was included in the 2004 WADA prohibited list with the following definition: "Gene or cell doping is defined as the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance".

In the 2011 list version, it was further articulated and specified as:

'"M3. GENE DOPING". The following, with the potential to enhance sport performance, are prohibited:

1. The transfer of nucleic acids or nucleic acid sequences;
2. The use of normal or genetically modified cells;
3. The use of agents that directly or indirectly affect functions known to influence performance by altering gene expression. For example, Peroxisome Proliferator Activated Receptor δ (PPARδ) agonists (e.g. GW 1516) and PPARδ-Adenosine Monophosphate (AMP)-activated protein kinase (AMPK) axis agonists such as 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) are prohibited.'

In the 2012 WADA list, the definition of gene doping was limited to only points 1 and 2, while the substances in point 3 (PPARδ agonists and AMPK axis agonists, GW1516 and AICAR) were moved to the class S4 of "Hormone and metabolic modulators".

In the 2013 WADA list, gene doping has a more precise definition in point 1: "The transfer of polymers of nucleic acids or nucleic acid analogues" [5].

3 Gene Engineering and Gene Screening

The evolution of 'gene engineering' started in the 1980s with the 'in vitro' production of active physiological substances, namely insulin, erythropoietin (EPO), growth hormone (GH) and insulin-like growth factor-1 (IGF-1), which were structurally very close to human hormones [6].

The evolution was extremely rapid. The first preparations of recombinant human EPO (rHuEPO) were obtained with the human EPO gene introduced into Chinese hamster ovary cell lines, and were responsible for subtle differences in carbohydrate structures. By using human kidney cells instead of hamster cells, Shire Pharmaceuticals introduced Dynepo in 2007. This new rHuEPO showed a molecular structure that was very close to the endogenous EPO. Because of this new structural analogy, Dynepo was mistakenly believed by some cheating athletes to be undetectable in the existing urine test at that time [7, 8]

Starting from two main research projects, HERITAGE and GENATHLETE, orientated to find genes responsible for endurance capacity in humans [9], many other performance-related genes were identified. Some genes and their polymorphism like the angiotensin-converting enzyme (ACE) coding gene, the (185-bp) gene code of the EPO receptor, the gene code of muscle creatine kinase, or the gene code of α2 adrenergic receptor positively correlated in some studies with an improved endurance capacity in runners [10].

The almost yearly review published by Rankinen and co-workers, hence called "human gene map for performance", updates the list of the discovered human genes connected with physical fitness phenotypes. To date, it includes up to 214 autosomal gene entries and trait loci, plus seven others on the X chromosome and 18 mitochondrial genes which showed a clear influence on fitness and performance phenotypes. Singular genes or particular genetic polymorphisms are connected with endurance phenotypes, or muscle strength phenotypes, or training response or exercise intolerance phenotypes [11, 12].

'Genetic screening' might become a selection method able to evaluate, according to individual genes or chromosomes patterns, the possible genetic predisposition of endurance- or power-orientated athletes. This distinctive feature was already documented by the study of PPARα gene polymorphism in a large cohort of athletes. These

subjects and their sedentary matched controls underwent DNA genotyping of mouth mucosal cells and muscular biopsy for a subgroup. Results showed that myotypology was linked to the PPARα gene polymorphism [13].

Some publications have also underlined the ethical aspect of a privacy violation connected with the use and the confidential management of information concerning individual gene mapping. Although not applied yet on a large scale, gene mapping of a targeted athletic population is feasible, as already documented. The talent identification at an early age, based on individual genetic predisposition, more than on observable external anthropomorphic characteristics around puberty, is not far from a conversion of sport from a field event to a laboratory schedule [14, 15].

A similar consensus on ethics and sport was reached during the 2005 WADA Symposium on gene doping in Stockholm, with the declaration that, "The use of genetic information to select for or discriminate against athletes should be strongly discouraged. This principle does not apply to legitimate medical screening or research" [16].

This risk of genetic screening abuse seems more likely since some papers underlined the important role of a single gene (*ACTN3, alpha-actinin-3*) in the expression of type II fast twitch fibres in speed performance [17, 18]. Although the contribution of this single gene is still debated, some commercial kits exist and offer the possibility of fast gene screening in athletes. Such a strategy, the interest in which has yet to be shown, does not appear ethical, especially among a young healthy athletic population [19].

According to the Council of Europe Bioethics Convention and the US *Genetic Information Non-discrimination Act*, tests on gene predisposition are ethically, or legally, applicable only for strict health reasons, and with appropriate genetic counselling, and are not for other capacity evaluation or employment purposes [20]. These rulings do not cover the screening of a priori healthy athletes for genetic diseases or conditions which could be revealed or made worse by sports practice [21]. A few typical examples are observed in some heart conditions (hypertrophic cardiomyopathy, long QT syndrome, arrhythmogenic right ventricular cardiomyopathy, Brugada syndrome, etc.), detected during pre-participation health examination, in which a strong link is observed with some familiar and/or genetic predispositions [22, 23]. In some of these conditions, a well-conducted genetic analysis makes possible correct risk stratification and some secondary preventive measures, including sports activity planning or withdrawal [24].

4 Gene Treatment and Gene Manipulation

Undoubtedly, gene therapy is likely to become an important tool for treatment of patients suffering from serious diseases. Some pathological conditions which could potentially take advantage of such kinds of treatment include congenital or acquired muscular dystrophies, diabetes, primary or secondary forms of severe anaemia (kidney failure, cancer), cardiovascular diseases and growth deficiency.

Unfortunately, another aspect should be considered about the possible use of gene manipulation. These future innovating therapies used as long-term treatment of some specific diseases will probably be simultaneously misused by athletes who cheat and their scientific advisers, in view of an artificial enhancement of physiological parameters and capabilities [25–27].

In many diseases, the medical art first aims to identify congenital or acquired deficiency or abnormal function for particular substances in the human body (proteins, hormones, enzymes, etc.). Then, the actual treatments consist of an attempt to balance the defective mechanism by external introduction of this deficient substance into the human body. This is the case, for example, with insulin, GH and EPO, which, while being insufficient, for example, in patients with diabetes, growth problems or anaemia, are externally produced in vitro or in vivo and subsequently injected into the human body [28].

The prenatal gene manipulation of embryonic stem cells is actually out of practical interest. By contrast, the postnatal intervention on somatic cells, by introduction of genetic material (DNA, RNA or genetically modified cells) into the body, is actually able to enhance or suppress the production of substances responsible for the patterns of the disease.

The progress of gene therapy is based on the identification of the genes responsible for the production of a specific substance/hormone/enzyme, followed by the introduction of this gene (transgene) into the human body. It is anticipated that this transgene will incorporate into the cell and will use the cellular machinery to synthesize the specified recombinant protein or hormone in vivo, directly inside the human body, leading to the production of a protein very similar to the missing or defective endogenous one [29–31].

In a similar way, athletes who are determined to cheat could try to use the same gene manipulations to improve their performance by increasing the production of some performance-related proteins. Consequently, being able to differentiate molecules produced from gene doping from their 'natural' counterparts is a real challenge [32, 33].

Several methods, including inhalation or injection, may be used to deliver the genetic material inside the body. Once the genetic material is included inside the nuclear mass of the cell, the gene DNA sequence will induce, through RNA, the production of the specific protein coded by the inserted genetic material. Various delivery methods,

together with different advantages and/or adverse effects, have been proposed:

- Direct transplant: With this technique, human cells are isolated from the body, genetically modified in vitro, screened and finally transplanted back into the donor. This method has received renewed interest since Medgenics recently initiated a phase IIb study using this EPO biopump in anaemic dialysis patients [34, 35].
- Transfection: This method involves non-viral transporters (liposomes, plasmids, lipid vesicles, plain DNA, DNA-protein complexes, naked DNA) [36]. The transport vector is generally easier and cheaper to prepare and is less subject to contamination than other methods. The gene material is injected and exerts only local effects. The immunogenicity of this method is low as well as the duration of action (days or weeks).
- Transduction with inactive viral vectors [adenovirus, adeno-associated virus (AAV), onco-retrovirus, spumavirus, herpes virus, lentivirus, semliki forest virus]: This seems, to date, to be the most effective method [37]. The duration of effects, which might also be systemic, is longer (months or years), but the preparation is more expensive, longer and has more risks. Indeed, the crippled inactive viruses are not pathogenic, but, compared with non-viral vectors, they show higher toxicity and immunogenicity, sometimes leading to rejection. Moreover, the risk of contamination with virulent or wild-type viruses during the preparation cannot be excluded, in spite of the contamination and safety tests conducted.
- Other techniques: Additional methods exist, such as microinjection, bioballistics (which uses small silver particles coated with the genetic material to be inserted into the recipient cell), and electro and chemical porations (which involve the creation of pores in the cell membrane so that the genes can be transferred easily) [38].

The most commonly encountered problems with gene therapy are [39–41]:

- the quality of the gene material: even if easily obtained and not expensive to produce in large amounts by bacterial cultures, the material needs to be purified afterwards by chemical or pyrogen substances and tested for safety;
- contamination with wild-type viruses;
- possible mutagenesis: this consists of a definitive change in the cell's nucleic acid (DNA or RNA) and the ability to induce genetic modifications of chromosome structure with malignant toxicity, within different diseases, including cancers;
- the potential and large immunogenic reactions induced not only by the introduction of different material (vectors or genes, or the gene–virus combination itself) into the body, but also by the internal production of a protein that sometimes slightly differs from a physiologically endogenous one;
- the risk of having non-targeted tissues activated by this transgene therapy;
- a deregulation or an activation of an oncogene secondary to a lack of control of the zone where the transgene is inserted;
- modulation of gene expression after introduction into the patient's body: in this situation, the amount and quality of the genetically produced substance are an issue as well as the duration of the autonomous effects;
- the risk of excessive or flawed production;
- environmental risks linked with the elimination of body fluids containing genetically modified viruses or their by-products;
- the possible integration of genetic material into germ cells, with genetic transmission of unpredictable traits to following generations.

5 Erythropoietin Gene Engineering and Therapy

EPO and its different chemical formulations or similar compounds (erythropoiesis-stimulating agents) are by far the most studied molecules. EPO was a revolution in pharmacology and clinical medicine, and provided to both patients and physicians a safe alternative to blood transfusion in cancer or kidney failure patients with severe anaemic status.

The documented history of Eero Mantyranta, a Finnish cross-country skier, winner of two gold medals (15 and 30 km) at the 1964 Olympic Games in Innsbruck, enabled scientists to discover that he was affected by a natural mutation in his genome [42]. A variation on chromosome 19p 1.3, connected with the EPO receptors, led to a deficit in feedback control on red mass and permitted a higher than normal production of blood cells and a higher haemoglobin level, which enabled increased oxygen delivery to body muscles.

Research undertaken since the 1990s identified a familial specific mutation, the dominant autosomic erythrocytosis, found in all members of one particular family or local ethnic group. This consisted of an increased sensitivity of receptors to EPO, with consequent higher numbers of erythrocytes (polycythaemia or erythrocytosis) [43–46]. A mutation of Janus kinase 2 genes was discovered as being able to produce some myeloproliferative disorders and, in particular, erythrocytosis [47].

As soon as rHuEPO had been introduced on the market to cure severely anaemic patients, it unfortunately became, for some unscrupulous athletes, the easiest way to improve their endurance performances and to maximize oxygen consumption illegitimately. This ergogenic effect is mediated by an enhanced haemoglobin level and probably also by increased angiogenesis [8, 48].

The ability of today's antidoping laboratories to identify different forms of erythropoiesis-stimulating agents produced in vitro [EPO, darbepoetin, novel erythropoiesis-stimulating protein, Dynepo, continuous erythropoietin receptor activator, peginesatide, etc.] [49–53] is limited by the biological half-life of these different substances [54]. In spite of the somewhat limited windows of detection in blood and urine of these substances, current laboratory techniques still show some good deterrent effects towards blood doping manipulation [55, 56].

The option of in situ gene delivery able to induce persistent EPO production 'in vivo' has been studied since the late 1990s. This was achieved through intramuscular injection in animals (mice and monkeys) of EPO gene encoded in adenovirus or encapsulated in plasmids or liposomes. As a direct consequence, a haematocrit increase was observed from 49 to 81 % in mice, persistent up to 1 year, and from 40 to 70 % in monkeys, persistent up to 3 months. Higher haemoglobin increases and longer persistence were even reported by other authors using an AAV gene [57, 58].

These exaggerated increases in haematocrit testify to overexpression of the transgene. This remains the most difficult challenge for scientists because of the inability to control the process of protein synthesis, which finally results in haemoglobin overproduction (polycythaemia or erythrocytosis). Some experimental studies in animals, using a gene code encapsulated in myoblasts (embryonic precursor cells), made possible EPO synthesis on demand, controlled by the intake of doxycycline/tetracycline or similar small promoter molecules, which worked as a switch on-off timer [59, 60].

Further progress was also based on knowledge of hypoxia-inducible factors (HIFs). These physiological transcription factors (or sequence-specific DNA-binding factors) are produced by an individual's cells, particularly in the kidney and heart. HIFs are produced in larger amounts in the case of hypoxic conditions, and are able to facilitate transcription of genes, to increase glycolytic enzymes and vascular endothelial growth factor (VEGF) and to induce secondary EPO overproduction by peritubular cells. For instance, a medical condition called Chuvash congenital polycythaemia is associated with reduced HIF degradation. This leads to an overexpression of EPO-related genes [45, 46]. The pharmaceutical industry has actually investigated some small molecules (FibroGen FG-2216 and FG-4592) that stabilize HIF through the inhibition of HIF prolyl hydroxylase (HIF-PH). Indeed, the inhibition of HIF-PH is able to increase the levels of HIFs, independent of the oxygen tissue levels, with enhanced expression of HIF responsive genes, EPO and/or VEGF. Synthetic HIF-PH inhibitors (FibroGen FG-2216), when orally administered to end-stage renal disease patients, significantly increased EPO levels [61]. The possible benefits of oral administration of these gene-expression modulators, counteracted by the risk of oncogenic vascular neogenesis, are still being investigated [62, 63].

A better control of gene expression was observed in mice with the use of Oxford Biomedica hypoxia response element, Repoxygen, a special EPO gene vector by which the EPO secretion, unlike the uncontrolled cytomegalovirus-carried gene, is activated only in the presence of anaemic or hypoxic conditions, and stops when the healthy physiological level is reached [64, 65]. As a means of increasing red blood cell numbers, and because of its self-regulating properties and its difficult detection, a former German coach attempted to order Repoxygen for the purpose of boosting the performance of his athletes. To the best of our knowledge the development of this gene therapy has been abandoned.

Unexpected adverse effects have sometimes been observed. These are easily explained by the EPO gene administration mechanism of action. First, the vector–gene complex might be immunogenic by itself, or because of the viral structure or the possible impurities contained in the combination complex. Second, when the genetic material is introduced into the body, there is a risk of DNA spreading to the whole organism. Some primarily non-targeted tissues (for instance, muscle cells) could then start to synthesize EPO, finally resulting in the production of a protein that is slightly different to normal physiological EPO. Indeed, the type of tissue which incorporates the EPO DNA and then produces the hormone might impose its own chemical signature (by altered post-translational modifications) on an EPO molecule that could differ slightly from the normal physiological one. This more or less 'biosimilar' molecule could then be recognized as abnormal, increasing the possibility of an immune response [66]. For instance, autoimmune anaemia was observed in macaques treated by EPO gene administration [67, 68].

These slight structural differences in genetically engineered EPO molecules are essential to the fight against blood doping. EPO abuse detection in athletes' urine samples is continuously improving, mainly using isoelectric focusing coupled with immunoblotting techniques [49]. For instance, EPO isoform synthesized by muscle cells, instead of renal peritubular fibroblasts, is detectable in the serum of macaques by isoelectric focusing, showing

different patterns in the same animals before and after gene transfer by AAV injected into skeletal muscle [69].

For some years, a new antidoping approach has been used to fight against doping. It is called the Athlete Biological Passport and is based on a longitudinal analysis of some selected biological parameters obtained from repetitive blood or urine samples from the athlete [70]. The purpose of this tool is to monitor the individual fluctuations of some selected parameters related to blood doping (haemoglobin concentration and reticulocyte count), which are normally subject to limited variations [71]. A mathematical and algorithmic model is then applied to detect very unlikely variations that could reflect either a pathological condition (to be proven by the athlete) or a blood doping strategy [72, 73]. This indirect tool is independent of the blood doping strategy used by athletes who cheat (erythropoiesis-stimulating agents or exogenous EPO administration or blood transfusion or EPO gene manipulation) [74, 75]. Although only the haematological module is actually implemented by some sports governing bodies, other modules such as the endocrine module (somatotropic, gonadal, and corticotropic axis) or urine steroid module are being studied.

6 Modulation of Gene Activity and Metabolism

An interesting and surprising approach to possible gene modulation came from an observation that peroxisome proliferator-activated receptor-gamma co-activator-1 alpha (PGC-1α), a transcription co-activator increased by exercise, was able to stimulate mitochondrial biogenesis and induce a more oxidative and less glycolytic metabolism during endurance training in slow-twitch fibres [76, 77].

Moreover, it was found that mice treated with resveratrol (a polyphenolic natural compound with antioxidant properties found in grape skins) raised their running time and oxygen consumption by induction of AMP-activated protein kinase, oxidative phosphorylation and mitochondrial biogenesis mediated by increased PGC-1α activity [78]. This also had a protective action against obesity, age-related diseases and insulin resistance [79, 80]. In addition, it was reported that transgenic mice with genetic expression of an activated form of PPARδ had a greater proportion of type 1 fibres, with higher oxidative capacity and increased endurance capacity [81]. Practically, it is reasonable to assume that the activity of PPARα, PPARδ, PGC-1α and PGC-1β might influence the messenger RNA (mRNA) expression in skeletal muscle fibres [82].

It has also been shown that some small molecules (such as GW1516) were able to directly activate the PPARδ pathway [81]. Others, however, such AICAR, after conversion into nucleotides, mimic the effects of AMP, activating AMPK in the nucleus. This exercise-like pathway activation promotes in this way the expression of oxidative genes by 'up-regulation' of PGC-1α and PPARδ, with a secondary increase of muscle metabolism. As a consequence, these molecules seem to show a greater physiological effect in trained animals, without excluding an autonomous effect on sedentary animals. In particular, GW1516 administration, while not working in sedentary mice, was able to increment up to 100 % the endurance capacity of individual mice when coupled with exercise. By contrast, 4 weeks of AICAR treatment alone, even in sedentary mice, was able to enhance running endurance by 44 % [83]. Animal experiments reported that these molecules could potentiate the physiological effects of exercise, in particular, boosting fat metabolism [84, 85]. They could also increase some cellular gene expression and thus the mitochondrial mass, as well as angiogenesis leading to improved endurance exercise capacity [86].

From a theoretical point of view, endurance athletes could benefit in terms of performance from the use of these substances, acting as 'exercise pills', by an amplified metabolic response, particularly on fatty acid oxidation [87]. However, the possible ergogenic effects in humans, if any, are likely to be irrelevant since such a doping strategy would need very large amounts of the expensive molecules in order to obtain any effects on the human body. Further, it is not clear whether AICAR delivery would produce any additional metabolic activation in muscle tissues of trained athletes where AMPK is already activated at a high level [88].

7 Angiotensin-Converting Enzyme Modulators

Genetic studies demonstrated, many years ago, a statistical correlation between endurance capacity in humans and the polymorphism of the ACE gene code [89]. Hence, a reduced ACE activity is linked to a higher maximum oxygen uptake, an increased exercise capacity and a greater response to training. A reduced ACE activity is responsible for a lesser conversion of angiotensin I to the vasoconstrictive angiotensin II and for higher levels of bradykinin (vasodilator). Numerous studies were conducted, with different ethnic groups, on the ACE 'insertion/deletion' polymorphism, frequently abbreviated as 'in/del' or 'I/D'. The possible prevalence of an 'ACE-in allele,' correlated with endurance capacity and evaluated in high-altitude-living indigenous Andean as well as in Kenyan, Australian and European athletes, did not show unequivocal results. Similarly, the 'ACE-del allele,' apparently connected with sprint/power performance and studied in Russian, British and Jamaican groups, led to various correlations [90]. Very recently, a human retrospective study showed that the ACE

I-allele modulates the muscle response to endurance training in cyclists [91]. The ACE I-allele carriers, when compared with non-carriers (i.e. ACE-DD genotype), demonstrated higher volume density of subsarcolemmal mitochondria and intracellular lipids. These enhanced adjustments corresponded to ACE I-allele–dependent up-regulation of muscle transcripts associated with glucose and lipid metabolisms.

Although not yet fully scientifically proven in a normal young healthy population, pharmacological ACE inhibition could improve exercise duration. The recently discovered ability of the angiotensin II receptor blocker telmisartan to induce biochemical and metabolic changes (e.g. mitochondrial biogenesis and changes in skeletal muscle fibre type), similar to AICAR, agrees with these previous findings [92].

8 Growth Hormone, Insulin-Like Growth Factor-1 and Myostatin

The anabolic effects of GH on muscle proteins and mass, together with the increases in the metabolism of carbohydrates and fatty acids, are well-known to unscrupulous athletes who use its human recombinant form. Furthermore, IGF-1, a protein mainly produced in the liver, stimulated by GH and a primary mediator of its action, but which is also actually produced as synthetic human recombinant IGF-1, is a doping substance. It is known to be able to prevent the age-related loss of muscle mass and to promote, in healthy humans, muscle hypertrophy and somatic growth, both of which occur by increasing the protein synthesis and the differentiation of satellite cells [93]. IGF-1 (produced in muscles as well) is also able to promote, after normal stress or injuries, muscle repair and relative hypertrophy through proliferation of satellite cells by division, fusion with muscle fibres and an increase in myofibrils and nuclei [94]. Lee et al. [95] observed an improvement in muscular mass (31.8 %) and force (28.3 %) after viral administration of IGF-1 gene (AAV injected in the posterior leg) in mice trained for 8 weeks by climbing with additional loads. This increase was higher with the association of IGF-1 gene and mixed strength-resistance training than with exercise alone or the IGF-1 alone. The muscle mass loss during the detraining period was significantly lower in rats treated with the IGF-1 gene, compared with untreated animals [95]. A possible medical use of IGF-1 would be to protect against age-related sarcopenia and, moreover, to help recovery from local muscle injuries (in the presence of adequate numbers of satellite cells in the muscle) [94, 96]. This could become an improper method for increasing body muscle mass, particularly when associated with strength-training exercises.

Myostatin is another important protein synthesized in skeletal muscle, and its gene expression has been widely studied because it acts as a regulator of muscle mass [97]. It is able to control and lessen muscle anabolism, by inhibition of satellite cell activation, muscle fibre hyperplasia or hypertrophy with a feedback mechanism [98, 99].

2Myostatin gene mutation or deletion in animals causes hyperplasia and hypertrophy with increased muscle mass in Blue Belgian and Piemontese cattle (20–30 %) and in mice (200–300 %). Indeed, in cattle breeds, the inherited truncated and ineffective form of myostatin allows uncontrolled muscle growth, and the animals have a lean, sculpted appearance, due to contemporary interference with fat deposition [100, 101]. Mice in which the myostatin gene has been inactivated show marked muscle hypertrophy [102]. This phenomenon was also reported with a dominant-negative form of myostatin receptor ActRIIB or with inhibition of myostatin receptors by follistatin or by myostatin propeptide (a mutant version of myostatin lacking the molecule's signalling portion) [103, 104].

Frequent sequence variation of the myostatin gene [105] as well as existing myostatin and follistatin polymorphisms [106] have been reported. These variations are likely to be responsible for the muscle phenotypes encountered in humans. Although never officially confirmed, to the best of our knowledge, a famous body building champion as well as a European weight-lifting champion were suspected of carrying such a genetic mutation. A few years ago, a particular muscle hypertrophy was observed, since birth, in a child with mutations in both copies of the myostatin gene with a typical myostatin deficiency, while the mother, a former professional athlete, had a lack of only one copy of the gene (no other family members were studied). The child, when 4.5 years old, was able to hold 3 kg with both extended arms [107].

Following these clinical and biological reports, extensive medical research has been conducted in order to produce small propeptides, or follistatin, or a mutant version of myostatin (both gene induced), which would be able to block the receptor from binding normal myostatin in muscular dystrophic patients [108]. Promising results from a study proposing to treat muscle-wasting disorders such as Duchenne muscular dystrophy have recently been published on a murine model [109]. These authors reported an effective myostatin blockade approach obtained with small interfering RNA and transcriptional gene silencing. Humanized monoclonal myostatin antibodies were also studied in dystrophic patients and/or in old patients with sarcopenia [110].

Unfortunately, myostatin blockade could potentially allow unscrupulous athletes to increase muscle mass rapidly. These antibodies, even if banned by antidoping rules,

could soon become a tempting way for dishonest athletes to improve their strength and power performances [111].

9 Vascular Endothelial Growth Factor

VEGFs are currently studied for gene treatment in some cardiovascular disorders (myocardial infarction, peripheral arterial disease). Their positive effect on angiogenesis is actually promising for partial recovery from ischaemic damage [112]. Gene transfer of VEGF with the common cold virus in humans might induce the production of new vessels, probably useful for patients with angina pectoris or other heart or vascular diseases requiring increased transport of oxygen to peripheral ischaemic tissues [113, 114].

In the future, enhanced capillary genesis within muscles and other body systems (cardiac, pulmonary, endocrine, etc.) might, unfortunately, become an alternative way of blood doping, by improved oxygen delivery to peripheral tissues.

10 The Health Risks of Gene Doping

Although theoretically helpful and relevant in some rare but serious human diseases, gene-based treatments are not free from sometimes unexpected and severe side effects. The transfer to humans of animal experiments is never easy, both because of different body sizes and species-specific reactions [115]. A French publication in 2008 reported that nine out of ten children with X-linked severe combined immunodeficiency were successfully treated with in vivo gene therapy using gamma retroviral vectors. Their immunological functions were significantly improved, and these young patients did not again develop the usual and often lethal infections [116, 117]. However, probably because of the insertion of a retroviral gene vector in the body cells, four out of nine successfully treated children secondarily developed severe T-cell leukaemia within 31–68 months, and one died [118].

An uncontrollable reaction to the vector was observed in another gene-based treatment where death was due to intravascular coagulation and multi-organ failure [119].

As mentioned above, the main risk with gene therapy arises from the uncontrolled activity of genes after their introduction into the body. This differs substantially from the direct administration of the substance, where the effects are limited to the administration period (for example, with EPO, GH and IGF-1) and quickly decrease after the cessation of treatment. Other risks with gene therapy are represented by a transgene being inserted in the wrong location in the DNA, or a gene modification on non-targeted cell lines, including reproductive cells, with a possible transmission of this transgene to descendants.

The possibility of uncontrolled red cell production, with no chance to stop the mechanism, is the main problem for EPO gene doping. An increased risk of hypertension, heart or brain vascular problems caused by high blood density remains possible. For example, in a similar way in young populations with familial and congenital polycythaemia, [45] a high incidence of early cardiovascular death (cerebral vein thrombosis or haemorrhage, peripheral thrombosis, congestive heart failure, etc.) has been reported [46, 47].

An overexpression of the IGF-1 gene could increase muscle mass beyond the expectations of athletes who cheat. Moreover, and as is seen in acromegalic patients where GH and IGF-1 circulating concentrations are very high, an uncontrolled development of connective tissue in some organs (heart, liver, lungs, etc.) is a possible consequence of IGF-1 gene overexpression. This could theoretically lead, for instance, to heart valvulopathies, heart failure, sleep apnoea (soft-tissue thickening), skin thickening and Raynaud disease. Lastly, the potent mitogen and anti-apoptotic effects of GH and IGF-1 are well-known, and an oncogenetic effect is more than a hypothesis with genetically stimulated and timely undetermined overproduction of these anabolic substances [120]. Similarly, genetically stimulated overexpression of angiogenic factors might potentially increase the vascularization of undetected tumours and contribute to their growth, as suspected with EPO and EPO mimetics [62, 63]. Unscrupulous athletes willing to manipulate their genes in order to inhibit myostatin may face a problem of poor control with exaggerated skeletal muscle hypertrophy. As previously reported with anabolic steroid [121] abuse, secondary connective tissues and skeletal overloads could increase the occurrence risk of osteo-articular injuries.

The immunogenic risk also cannot be ruled out. It is particularly prevalent when using the adenovirus as a vector, but it is also frequently caused by impurities persisting after the preparation and production of vectors [66]. Furthermore, a subtle structural difference of a genetically produced protein by non-targeted tissues could trigger pathological reactions such as those observed in autoimmune anaemia of EPO gene-treated macaques [67, 68]. Although unlikely, the possibilities that viral vectors might suddenly acquire a high virulence or produce a new virus mutant can never be excluded [122].

Admittedly only germ cell mutations (and not somatic cell changes) could be transmitted to following generations. In the event of this theoretically unlikely phenomenon occurring, some serious ethical and legal concerns would be raised [123].

11 Strategies for Detection

While the banned doping substances or methods are detected by direct and indirect methods, the same approaches are not so easily applicable in the case of gene doping. This is because of the particular conformity of genetic material to the physiological DNA, which makes both non-invasive and direct detection methods difficult [124]. The attempted detection methods of vectors, even when indirectly based on the immune response of the body (for example, to viral vectors such as adenovirus or others), are often unable to discriminate between natural infection and artificial introduction of the virus. However, some recent detection methods have been successfully tested on macaque white blood cells. Indeed, the authors were able to detect recombinant AAV (rAAV) viral vectors by standard real-time polymerase chain reaction (PCR) assays in macaque white blood cell genomic DNA up to 57 weeks after intramuscular injection, with a very low risk of false positives or false negatives [125, 126].

Identification in body fluids of the small molecules (antibiotics such as rapamycin, doxycycline and tetracycline or other substances such as antiprogestins) used as switch-on or switch-off promoters of inducible gene activity provides indirect evidence of gene manipulation when not justified or authorized for medical treatment. However, it remains problematical, since some of these drugs are commonly used in medical practice and are not included in the WADA list of prohibited substances and methods.

The unlikely 'direct' detection of vectors or locally injected genes could be possible only if:

- the analysis is conducted early enough after administration;
- in the case of injection, the local treatment site is known;
- the athlete accepts invasive procedures (such as biopsy), which is unlikely.

Labelling the gene or vectors with specific markers added by the manufacturers, as proposed for agricultural transgenic production, is a theoretical option. However, such labelling is not free of risk and may increase immunogenic and technical risks with the introduction of an additional component. It may compromise treatment efficacy, and it is uncertain that the pharmaceutical companies will accept this procedure and bear its additional cost. Last but not least, any illegal production of the transgene by a rogue laboratory could render this detection strategy completely inefficient. For all these reasons, this 'labelling' option as a doping detection method has been abandoned.

On the other hand, the above mentioned study of Lasne et al. [69] was able to identify, in macaque serum samples, EPO isoforms produced by injection of AAV encoding EPO gene and controlled by doxycycline as a promoter. It was possible to differentiate post-translational features (observed by double-blotting following isoelectric focusing on serum) in the protein produced by skeletal muscle compared with the one obtained from fibroblasts of peritubular kidney tissue.

The misuse of the IGF-1 gene or other mechano growth factor genes, while being locally injected in the usual physiological production site of the endogenous forms, will produce similar isoforms only at the local level. Consequently, any potential detection could be via analysis of a sample collected via local biopsy, rather than by the easier and usual method of body fluids collection.

However, a promising method of direct detection of EPO transgene (complementary DNA used as a transfer gene, with a subtle difference from the endogenous genomic DNA) has been described. It used particular PCR assays in order to amplify selectively in the blood the very minimal amounts of possible transgene DNA [127, 128]. Similarly, Ni et al. [125] recently showed that intramuscular injection of a conventional plasmid or rAAV vectors results in the presence of DNA that can be detected at high levels in blood before rapid elimination, and that rAAV genomes can persist for several months in white blood cells. These collaterally transfected white blood cells could thus be used as surrogate markers for gene doping.

Some new alternative methods are now evaluated [39]; generically called 'transcriptional profiling,' they aim to detect changes in protein levels compared with basic measured physiological levels. This would, of course, require simultaneous and repeated measuring of thousands of proteins from gene expression (transcriptomics) to protein profiling (proteomics) and their biochemical results (metabolomics) [124].

Transcriptomics, by microarray technology, seems able to identify subtle changes in thousands of genes. It is, however, not certain that a single gene modification could lead to detectable differences and discriminate between a physiological and artificial (gene doping) condition. Proteomics is based on analysis by two-dimensional gel electrophoresis and capillary electrophoresis or two-dimensional liquid chromatography able, after separation, to identify and quantify an enormous number of proteins with mass spectrometry. Some specific groups of biomarkers could probably be targeted and used for antidoping purposes. Metabolomics, while analysing low weight targeted metabolites, could provide evidence for suspicious metabolic responses to artificial stimuli. By specifically revealing the impact of a doping substance or method, this interesting antidoping approach could overcome some of the limitations encountered with doping agents with short half-lives.

Practical limitations to these methods include the necessity of frequent and regular profiling of a single athlete, with consequent practical and laboratory limitations. In fact, the main difficulty of indirect '–omics' methods based on the changes of gene expression in blood cells (transcriptomics) and blood or urine (proteomics and metabolomics) would be the determination of which levels should be considered normal and which changes might give unambiguous evidence of doping. Indeed, individual genetic profiles, exercise, diet, ethnicity or environmental factors could lead to physiological changes on levels of specific gene products. These various confounding factors and their effects should be addressed by studying sufficiently large and heterogeneous sedentary and athletic populations during the development phase of the antidoping method. Then abnormal longitudinal changes of mRNA, proteins or metabolites could attest to a gene doping practice. However, some athletes who don't cheat might also carry an innate genetic feature or mutation (undiagnosed pathological condition) which could also alter their individual profile.

Radionuclide imaging, both positron emission tomography and single photon emission computerized tomography, have been tested in clinical studies on gene transfer in humans, but the use in detection of sport manipulation appears irrelevant from a practical point of view [129, 130].

So far, the most likely situation regarding modern doping seems to be linked more to biotechnologically assisted doping than to real gene doping. These scenarios are linked to the misuse of either gene modulators such as GW1516 or AICAR or myostatin modulators (anti-myostatin antibodies). These molecules, which appear to be available already on the black market, represent a serious and real threat to fair play in sport [131]. GW1516, a fully synthetic compound, can be detected in plasma by protein precipitation, centrifugation and analysis by liquid chromatography mass spectrometry of the supernatant [132]. Studies on metabolism and renal elimination are showing the development of a valid detection test in urine [133, 134]. AICAR is, on the other hand, a natural intermediate of biosynthetic pathway of purines. Hence, its physiological presence in urine samples of healthy humans is as well-known as the variability of its concentration due to various physiological conditions or nutritional status. Both endogenous production and exogenous intakes are measured in blood or urine by liquid chromatography and mass spectrometry. Consequently, analytical studies are being conducted to quantify the amount of a physiological production and its confounding variables in athletes (gender, type of sport, and different seasonal periods) [135, 136].

12 Conclusions

Gene therapy is actually limited to some particular and serious medical conditions, but the related research has helped in discovering new molecules and pathways of human biology. In the future, practical applications of this research could hopefully help the recovery from some more genetic diseases, with a return to normal life and physical activity. Similarly, the local therapeutic use of some kinds of genetic material could improve and shorten the recovery time from muscle, bone, joint or tendon serious injuries. As a result, it could become, in future years, a challenge to distinguish between an ethically accepted medical therapy, improving recovery from an injury, and a banned gene doping practice [15, 137]. In this situation, the sports medicine community will have to work closely with WADA in order to amend and fine tune its genetic antidoping rules. Similar to past decades, scientific knowledge as applied to sports physiology could become an interesting tool for improvement of performance, by artificial manipulations of individual genetic features. The black market is always ready to provide unscrupulous athletes with substances which have neither completed all phases of clinical study, nor even proved their ergogenic value in high-level athletes. Although the high-tech genetic industry has so far paid little attention to sport, some potential athletic cheats and their entourages have already shown a great interest in some of the above-mentioned substances (metabolic or gene modulators) in order to stimulate endogenous metabolic processes. Whether a single gene manipulation can significantly improve physical performance is still being debated. However, it appears from the present review that some particular genes or cellular pathways are sensitive, since they are comprehensively linked to the modulation of some components of athletic performance. The sports science community is now engaged in a difficult task: they must try both to prevent adverse health effects and to detect any possible performance-enhancing gene manipulations. Last but not least, the politics of gene doping detection will have to be a sufficient deterrent, partly because of its costly research, development and implementation. Unfortunately, it will not be possible to measure the outcomes of such a difficult commitment for several years.

Acknowledgments The authors acknowledge Ms Lindy Castell for her valuable contribution in reviewing the present manuscript.

Conflict of interest The authors declare no funding and no conflict of interest.

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