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New trends in gene doping

By Giuseppe Fischetto

ABSTRACT

Advances in the science of genetics are leading to more and more therapeutic options for many genetic and acquired diseases but almost inevitably the techniques of gene therapy are attracting the attention of sports cheats as well. For example, it will soon be possible to manipulate the human genome to stimulate increased production of substances currently used for doping within the human body (in vivo), making detection increasingly more difficult and problematic. Other techniques with legitimate medical applications could be hijacked as well and each represents a threat to health and sport. Gene doping, therefore, is the newest front in the battle for safe and fair sport. The author, a member of the IAAF Medical and Anti-doping Commission, provides a brief history of the application of genetic science to sport, an explanation of techniques and related issues in medical gene therapy, brief descriptions of the main forms of gene doping that could be used in the future, the health risks of various forms of gene doping and a discussion of the issues related to enforcement of the regulations against gene doping in sport.

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Introduction

The mapping of the more than 30,000 genes in the human genome and the knowledge it has provided, while initially helpful for understanding and prevention of some human diseases, may soon become useful in therapeutic strategies as well. The human genetic map could, in the future, make possible therapies that lead to the improvement of physical, endocrine or metabolic functions. These in turn could provide significant benefits to individuals with serious genetic or acquired diseases, such as muscular dystrophy, diabetes, endocrine defects, cardiovascular problems, haematological diseases (haemophilia, thalassemia, sickle-cells anaemia) and more.

However, when imported into sport medicine, this knowledge might lead to genetic techniques for artificially enhancing sport performances, in other words "gene doping". These techniques, of course, would be illegal from a sporting point of view and medically unethical, not least because of the related health risks. Unfortunately, they may prove difficult to detect[9, 23]. The author of this article strongly supports current anti-doping regulations and condemns the use of all proscribed methods to artificially enhance performance in sport.

The purpose of this article is to provide an overview of the current situation and thinking with regard to gene doping. It provides a brief history of the application of genetic science to sport, an explanation of techniques and related issues in medical gene therapy, brief descriptions of the main forms of gene doping that are currently used or could be used in the future, the health risks of various forms of gene doping and a discussion of the issues related to enforcement of the regulations against gene doping in sport. It is hoped that the provision of information in this form will contribute to the fight against doping by showing that sport authorities, the medical profession and the scientific community are addressing the issues related to this very difficult problem. It is also hoped that the information provided here will assist anyone involved in sport to make informed and ethically sound decisions.

Historical evolution

The first area where genetic science has been applied to sport is what is called "genetic engineering". Since the 1980s, it has been possible to artificially produce substances normally produced by the human body, such as insulin, EPO (erythropoietin), GH (or HGH, human growth hormone) and IGF-1 (insulin-like growth factor 1), which can be used for doping purposes. The techniques used are not manipulation of the genome in a human body; they are, instead, the production of human-like hormones *in vitro*[14].

Gene activated EPO, for example, might be considered an initial, primitive step of this approach to gene manipulation. Preparations of rHuEPO can be obtained from the human EPO gene introduced into rodent cell lines, a process that is responsible for slight differences in the carbohydrate structure, which permits identification of the product when misused in doping cases[1]. However, Dynepo has developed an equivalent process, called "gene activation", which works *in vitro* on human cells. This should make it possible to obtain EPO that is quite similar to endogenous EPO and for this reason not easily detectable[11].

The next area of investigation that is starting have application in sport is "genetic screening" and its role in the process of selecting and training athletes. At present, talent identification is largely based on observable external anthropomorphic characteristics. However, by studying the genome of an individual at an early age, it may be possible to discover an individual's genetic predisposition to a specific sport or event. This makes it possible to train athletes with the most specific methods or instruments for their personal genes or chromosomes patterns[36]. In the future, it might even be possible to screen elite athletes with a simple blood genetic examination[24]. The actual level of research in this field is still very far from a realistic possibility of practical use. Nevertheless, gene mapping, which is not formally banned at the moment, might in future create some real problems, in particular the possible unethical violation of individual privacy.

Two major research projects in the area of genetic predisposition, HERITAGE and GENATHLETE, are currently trying to find the genes responsible for endurance capacity in humans. In recent years, many discoveries have been made, the most significant of which are: a polymorphism of the gene code for ACE (angiotensin converting enzyme), the gene code (185bp) of the EPO receptor, the gene code of CKMM (muscle creatine kinase), and the gene code of the alpha 2 adrenergic receptor (ADRA

2A). All of these have been connected with improved endurance capacity in runners[30, 36].

But current research work goes even further. In a review, published annually by Rankinen et al, we can follow the updating of the list of discovered human genes connected with related fitness phenotypes. This amazing list is really a "gene map" of human physical capability. At present, there seems to be 109 autosomal genes, two genes on the X chromosome and 15 mitochondrial genes that influence fitness and performance. Moreover, it is possible to connect singular genes or genetic polymorphisms with endurance phenotypes, muscle strength phenotypes, and training response or exercise intolerance phenotypes[28, 29].

A third area of application of genetic knowledge is "gene therapy", which is the direct manipulation of human cells *in vivo*. This concept, which is the next research step in both medicine and physiology, is possible in the following ways[36]:

1) Prenatal gene manipulation of stem cells, directly on sexual gametes or fertilised ovarian cells, which could permit the treatment of genetic anomalies, whose location and genetic defect on the chromosomes are known. (Note that these techniques are not presently allowed worldwide for legal and ethical reasons.)
2) Postnatal gene therapy on somatic cells, that is the introduction of genetic materials (DNA, RNA, genetically altered cells) into the body or body cells in order to suppress or enhance the production of disease related substances.

In the remainder of this article, we will leave aside discussion of prenatal gene manipulation, and all the related legal and ethical issues, and will examine only the postnatal genetic approach.

Unfortunately, the possibilities created by gene therapy to correct certain defects of the human body and improve the medical circumstances of really ill humans might also become methods to improve some body features, thereby permitting illegal and unethical performance enhancement in athletes. In other words, "gene doping".

Gene therapy in medical practice

The methods to deliver the genetic material into the body are:

1) Transduction, which involves viral vectors (adenovirus, retrovirus etc);
2) Transfection, which involves non-viral delivery systems (liposomes, plain DNA, DNA-protein complexes);
3) Direct Transplants of genetically modified cells, initially isolated from the human body and then transplanted back.

Each of these methods has some advantage, and some adverse effects[34].

Usually the genetic material is introduced by local injection or by aerosol. The substances produced are quite similar to the natural proteins. The effects of an injection may be only local (MGF) or it may be systemic if the produced protein goes into the circulation (EPO). The duration of the effects may be short (days or weeks) or long (months or years), depending on the quality of gene vector and if any promoting or inhibiting substances are used[13]. After the introduction in the patient, the DNA (gene) is able to produce RNA, which will subsequently synthesise the relevant protein.

Viral vector delivery is the most effective and the most expensive. The crippled, inactive viruses are not pathogenic but compared with non-viral vectors they have a higher toxicity and immunogenicity, which sometimes leads to rejection[13]. Moreover, they are difficult to prepare and subject to contamination with wild-type viruses, and therefore might be very dangerous for patients. Non-viral vectors are locally effective, have less side effects, are easier and less expensive to prepare than the viral method and they have less risk of contamination.

A key issue in gene therapy is the "quality" of the artificial gene. Normally the DNA of

the required gene can be easily produced in large quantities using bacteria. After production, it is purified but this process is not always 100% reliable. Chemical impurities, pyrogen substances and virulent viruses are the most frequent problems.

A second issue is the ever-present possibility that the host genome may induce a "mutagenesis", which is a definitive change of a cell's nucleic acid (DNA or RNA). A change to the DNA is capable of inducing genetic modifications, which may also involve the chromosome structure and become the cause of high or malignant toxicity, inducing diseases or cancer.

A third issue is the possibility that the tissue programmed by the introduced genetic material to synthesise a specific physiologic protein, will produce a slightly different protein, which might induce a large immune response. For example, the EPO produced by muscular cells might be a little different from EPO produced by kidney[7, 10].

A fourth issue is the unexpected consequences of gene therapy. For example, adverse effects have been observed when certain gene-virus combinations are used for specific disorders and have an undesirable effect on other systems of the body.

The biggest problem with gene introduction, however, is the inability to control the resulting process inside the body. For example, animal experiments have shown that the use of the EPO gene, introduced with an adenovirus, can increase the Ht (haematocrit) 70-80% in monkeys and mice, an uncontrolled and dangerous over-expression of the gene[7,37]. It is possible that because genetic EPO therapy works in a systemic manner on the whole organism, additional tissues that are not normally programmed to synthesise erythropoietin start to produce abnormal EPO protein thereby increasing the chance of an immune response[10].

Currently, there is a lack of mechanisms to enhance or decrease the production of the desired proteins and the efforts of researchers are now focused in this direction[26, 34]. Using a particular non-viral vector in experiments with mice, British researchers were recently able to increase the blood parameters Hb (haemoglobin) and Ht to a physiologic level only, without any overproduction of EPO[4]. Other scientists are trying to find medicines able to activate or stop, according to the necessity, the gene function in the body[26].

Forms of gene doping in sport

EPO

The necessity to improve the production of red blood cells in the treatment of many human illnesses (cancer, chronic kidney failure, severe anaemia) and to assist in chemotherapy treatment has lead to the development of investigations of EPO (erythropoietin) into one of the largest research fields in medicine. The aim is to find an alternative solution to blood transfusions or the use of exogen erythropoietin.

This, of course, attracts the attention of those seeking methods to artificially increase the oxygen transport capabilities of healthy athletes. The use of EPO, including the possible use of the so far undetectable Dynepo, in athletics and other sports is already a recognised problem. It is not difficult to imagine what could happen in a few years when it becomes possible to insert the EPO gene directly into the human body with controlling mechanisms, and thereby induce the endogenous production of quite physiologic EPO and red blood cells[1, 4].

The documented history of Eero Mantyranta, a Finnish cross-country skier who won gold medals at the 1964 Olympic Games in Innsbruck, is very interesting in this respect. This athlete was affected by a natural mutation in his genome. An anomaly on chromosome 19p 1.3, which is 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. This gave Mantyranta a greater capability to conduct oxygen to his body muscles.

Research in this area has led to the identification in the 1990s of a familiar specific

mutation of the EPO receptor, which was responsible for the dominant autosomic erythrocytosis found in all members of one particular family[8, 15]. The subjects affected by this defect had an increased sensitivity of the receptors to EPO and consequently they produced a higher number of erythrocytes (polycytemia or erythrocytosis), with result being higher levels of Hb and Ht.

An advanced study has been conducted into the possibility of controlling the expression of the AAV (Adeno Associated Virus Vector) EPO gene in mice by using OBHRE (Oxford Biomedica Hypoxia Response Element). OBHRE works as an optimised hypoxia-responsive promoter, permitting a physiologic self-limitation to the EPO secretion process[4]. The expression of the gene was controlled better by the OBHRE (which worked only in the presence of hypoxia) than by an uncontrolled cytomegalovirus promoter, which induced an over-production of erythrocytes.

In other work, the use of incapsuled myoblasts (embryonic precursor cells) to deliver the gene code, has recently been found to be capable of creating a tetracycline/doxycycline dependent mechanism of control[6, 31, 35]. The myoblasts are introduced into the body where they proliferate and then become an integrated part of the muscle, with the possibility of long life and highly effective function. The transplantation of myoblasts into mice previously treated with retroviral EPO gene vectors containing a transactivator able to recognise a promoter inducible by the administration of tetracycline, permitted a controlled "On/Off" switching of EPO secretion.

IGF-1 and MGF

It is known that IGF-1 (insulin-like growth factor 1) can prevent the aging-related loss of muscle mass and in healthy humans promote muscle hypertrophy by increasing the protein synthesis and satellite cells proliferation[21]. Sweeney and others were able to improve the muscular mass (31.8%) and force (28.3%) in mice using a viral administration of the IGF-1 gene. This increase was the result of the association of the gene with resistance training, and was stronger than either the exercise alone or the gene alone[17].

Whilst in medicine the use of IGF-1 and gene therapy to stimulate its production may in the future prevent the aging-related loss of muscle mass and represent an alternative solution for treating local muscle injuries (if satellite cells are sufficiently present in the muscle), those seeking ways to artificially increase the muscle mass of athletes might, as an unusual method, also seek to make use of it, together with exercise[2, 3].

Similar to IGF-1, MGF (mechano growth factor) and gene therapy to stimulate its production may have an ethical medical application as well as the potential for misuse in sport. MGF might prove be useful for its local effects on muscles, ligaments, bones, condral, meniscal and other tissues, as it helps to repair injuries in short time. However, we must be aware that this factor might also be used in doping practice to enhance the size and strength of specific muscular areas.

Myostatin

The protein myostatin is normally able to control the increase of muscular mass by inhibition of satellite cell activation[18, 22]. In an American body builder, Flex Wheeler, a mutation of the myostatin gene, which permitted a particular muscle hypertrophy, was found[36]. Some myostatin gene anomalies are able to increase the muscular mass in animals without limit. The myostatin inhibition with follistatin or mutant receptors is able to increase the skeletal muscle mass, both for hyperplasia and hypertrophy[12]. Therapy with myostatin inhibitor genes might be useful for treating muscular diseases, particularly dystrophies, but it could also be an "interesting" approach for athletes seeking to gain muscle mass[5].

VEGF

VEGF (vascular endothelial growth factors) increases the growth of blood vessels in the muscles. Genetic transfer of VEGF, using the common cold virus, has been shown to increase the production of new vessels in humans. This therapy may have an ethical

medical use in treating patients with angina or other vascular diseases. However, athletes may, as an alternative to blood doping, try to use the developed techniques to increase the transport of oxygen to peripheral tissues[20, 27].

Leptin

This hormonal substance is related to the feeling of hunger. Manipulation of the related gene might be useful for losing weight, and could give benefit to sport cheaters[25].

Endorphins

These substances inhibit pain. Therapies focused on the genes encoding these substances, might be used in an attempt to reduce the pain associated with lactic acid in exhausting efforts or, working as analgesic peptides, to reduce the inflammatory pain connected with exercise stress[19].

The health risks of gene doping

In all forms of gene doping employing viral vectors, the immunogenic risk will always be present. The risks are associated with the gene-virus complex itself, with the production process and, sometimes, with immunogenic products, which can be the source of auto-immune responses[33]. Further, the use of viral vectors, is always subject, without any prediction, to the possible production of recombinant viral vectors with high virulence, or of new virus generation.

In the case of EPO gene doping, the biggest risk is uncontrolled red blood cell production. With no possibility to stop the mechanism, the individual is subject to greatly increased possibilities of peripheral thrombosis and cardiovascular problems, caused by the high blood density, affecting the heart and or the brain with potentially fatal consequences[32].

Over-expression of the IGF-1 gene or the myostatin inhibition gene could lead to an unintended degree of increase in muscle mass, which without parallel development of the bones, tendons and connective tissues could result in critical osteoarticular problems. Further, the effects of IGF-1 on other body organs (hearth, liver, lungs, etc), appears to be seriously dangerous and there is an increased possibility of tumour induction, or expansion, as there is with the use of MGF or VEGF.

Contact with, manipulation of, and even environmental exposure to the body fluids (urine, blood, saliva) of humans treated with gene therapy, may be a significant problem in the future. This is not such an issue for controlled patients, but athletes using these therapies for doping purposes are unlikely to be controlled[13].

And finally (for this list), there is the possibility of the transmission of genetically modified traits, capacities and problems to future generations. This would be a devastating situation, with ethical and legal implications. Whilst we can say that, unlike germ cell mutations which are transmitted, somatic cell changes should not be passed on, we have to add that nothing is completely sure in this area[37].

Rules and possibilities for the detection of gene doping

A prohibition against gene doping was inserted in the WADA (World Anti-doping Agency) prohibited list for the first time in 2003. The 2005 version of the list reads: “The non-therapeutic use of cells, genes, genetic elements or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited”.

Easy lecture, but difficult application!

How will gene doping be detectable? It has to be said that, at the moment, there is no proven effective method available. It is possible through genomic and proteomic analysis to identify therapeutic genes when they or, better, their vectors are “labelled” with specific markers[13]. Proteomic analysis (which involves simultaneous separation by bi-dimensional electrophoresis and identification by mass spectrometry connected with sophisticated computerised analysis) is seen by some as promising. The technique seems to make it possible to rapidly check thou-

sands of proteins. It is proposed that eventually all thereputic genes and vectors are marked and that there be frequent examination and individual serum protein profiling of athletes using proteomic techniques. However, the procedure would not be easily practicable. Morevover, the possibility raises many difficult issues:

- Scientists, sport organisations and the pharmaceutical industry would need to agree on any procedure.
- The additional risk of gene anomalies caused by an inserted protein marker, either on the gene or the vector, might not be only a supposition and there is the possibility that subsequent immuno-reactivity or the production of altered and unexpected proteins might be enhanced.
- The marked proteins or cells could be found only by muscular or tissue biopsy, and athletes to be controlled must be available for this procedure!
- The site of an injection of the vector-gene complex is not easily detectable; it could be local, on target organs, as for MGF or IGF, but it is occasional as for EPO.
- The use of indirect methods, which measure effects, for example elevated levels of Hb or Ht, might be contested because it would not be possible to distinguish between natural "endogenous" production of high levels of EPO and gene doping. The use of current regulations might not be practicable and applicable in this context.

However, progress on the scientific front is being made. Very recently, in September 2004, Lasne, Chenuaud et al, published new research about the possibility of discovering EPO gene doping. The supposition is based on the presence of a subtle difference between the primate EPO, which is produced by the renal peritubular fibroblasts, and the EPO produced by skeletal muscle after gene injection. By double-blotting following isoelectric focusing on serum, they were able to see a clear difference between EPO produced by macaques without and with gene transfer. Moreover, the analysed EPO patterns were significantly different in the same animal when examined before and after gene transfer[16]. The EPO physiologically synthesised in non-treated animals was quite different compared with EPO from gene treated animals. This appears to be an encouraging possibility for detection of some EPO gene doping.

Medical ethics

Finally, we need to address this situation from the point of view of medical ethics. Suppose an effective gene therapy has been legitimately applied to meet a medical need. For example, a patient affected by genetic or acquired diseases, and successfully treated by gene therapy, might ask to compete. What would be the correct answer? WADA rules ban the "non-therapeutic" use of genes or genetic elements or cells. Formally, the rule does not ban "therapeutic use" for the treatment of disease or injuries.

In another example, the tissue growth factors are a part of the current treatment for muscle, bone, ligaments and tendon injuries to which athletes and other sportsmen are often prone. On this line, the local transfer of genes producing "growth factors" might be the most useful therapeutic approach. The same assertion might be valid for many other diseases. Will such medical interventions be permitted or not?

And these are just two examples. The prospect of therapeutic management of genes in sport opens an enormous field for discussion on the availability and ethics of scientific medical instruments of therapy. Would it be ethically acceptable to permit or ban some kinds of gene therapy, at least locally? How would it possible to distinguish between local or systemic use? Will it be possible to obtain a "therapeutic use exemption" for justified gene therapy or not?

The ethical question is spontaneous and immediate. The answer is open!

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