

Review

Doping in the recombinant era: Strategies and counterstrategies [☆]

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Abstract

Advances in recombinant DNA technology have created one of the most powerful weapons in the current doping arsenal: recombinant proteins [Sweeney HL. Gene doping. *Sci Am* 2004;291:62–9; Unal M, Ozer Unal D. Gene doping in sports. *Sports Med* 2004;34:357–62]. Recombinant erythropoietin (EPO) and human growth hormone (hGH) are currently being abused but are fortunately detectable either directly by employing isoelectric focusing and immunoassays or indirectly by assessing changes in selected hematopoietic parameters. The detection is technically demanding due to the extent of similarity between the recombinant proteins and their endogenous counterparts. Another issue facing detection efforts is the speed and conditions at which blood samples are collected and analyzed in a sports setting. Recently, gene doping, which stemmed out of legitimate gene therapy trials, has emerged as the next level of doping. Erythropoietin (EPO), human growth hormone (hGH), insulin-like growth factor-1 (IGF-1), peroxisome proliferator-activated receptor-delta (PPAR δ), and myostatin inhibitor genes have been identified as primary targets for doping. Sports clinical scientists today are racing against the clock because assuring the continued integrity of sports competition depends on their ability to outpace the efforts of dopers by developing new detection strategies.

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Introduction

Doping in various forms has always been a major problem in competitive sports, and it has always been the role of clinical scientists to constitute the main line of defense against doping by developing reliable and practical detection methods. Today, recombinant protein abuse is an evolving form of doping that poses a strong challenge to clinical detection. The World Anti-Doping Agency (WADA) prohibits the use of recombinant proteins which are classified under substance doping [2]. Recombinant proteins threaten to help make good on a promise of the “super athlete” at the cost of sports ethics.

These doping agents are exceptionally threatening to sports and challenging to clinical scientists due to a combination of their incredible performance enhancement potential and the fact that they are essentially the same as their endogenous counterparts. As detailed below, there are documented incidents of abuse of recombinant proteins, and there are also established methods for detecting some of them. However, this remains one of the most challenging tasks in doping detection.

Candidate doping recombinant proteins and current detection methods

The two main recombinant proteins that are currently abused are recombinant EPO (rEPO) and rhGH [3]. Initially, immunoassays generally yielded rather poor results in detection of recombinant proteins since the antibodies used were unable to distinguish between endogenous and recombinant proteins [5]. Table 1 summarizes the main methods available for detection of rEPO and rhGH.

Erythropoietin (EPO)

Increasing oxygen delivery to tissues is important to optimize muscular activity and improve athletic performance, particularly in terms of endurance. Several methods have been shown to increase oxygen delivery to tissues including altitude

and hypoxic rooms, blood transfusion, and treatment with erythropoietin (EPO).

EPO is a 165-amino-acid (34 kDa) glycoprotein synthesized by the kidney in response to low blood oxygenation. EPO stimulates erythropoiesis through action on erythroid progenitor cells. The carbohydrate content of EPO is about 40%, a feature which contributed greatly to the detection of rEPO. Glycosylation of EPO is species and tissue-specific and critical for its biological activity. Recombinant and endogenous EPO isoforms have different glycosylation patterns [5]. rEPO is widely used to treat anemia caused by chronic renal disease. EPO was the first recombinant hematopoietic growth factor produced and has been available commercially as a recombinant protein drug since 1989 [5]. Several types of rEPO are commercially available including: Epoetin alpha (Eprex, Janssen-Cilag), Epoetin beta (Neorecormon, Roche), and Darbepoetin alpha (Nespo, Dompè) [5,6]. It is estimated that doping using rEPO was being implemented by 3–7% of the best athletes of endurance sports [7]. Side effects of EPO include hypertension and thrombotic cardiovascular and coronary events. The Sydney 2000 Olympics marked the beginning of the use of effective methods to detect injected rEPO.

Because EPO levels fluctuate over time and among individuals, measuring EPO concentration alone may not be useful to detect doping. Potential abuse of rEPO can best be detected by measurement of five hematopoietic parameters. These are: concentration of serum EPO, hematocrit level, percentage of reticulocytes, percentage of macrocytes, and concentration of serum-soluble transferrin receptors (sTfr) [6,7]. The reference values for these parameters were found to vary by gender, ethnicity, as well as altitude [8].

Two models were developed based on the behavior of each of the 5 parameters during and after controlled treatment with rEPO [9]. The “ON” model is applied during or shortly after rEPO treatment, whereas the “OFF” model is used weeks after termination of treatment [6,9,10]. A simpler approach employs only a combination of hemoglobin level, concentration of serum EPO, and percentage of reticulocytes; it was found to have higher sensitivity in cases of low dose rEPO abuse [6]. For the “ON” model: “ON” score = hemoglobin + 9.74 ln (EPO); OR hemoglobin + 6.62 ln (EPO) + 19.4 ln (sTfr). For the “OFF” model: OFF score = hemoglobin – 60 (reticulocyte percentage)^{1/2}; OR hemoglobin – 50 (reticulocyte percentage)^{1/2} – 7 ln (EPO) [11].

If these parameters were unusual, isoelectric focusing (IEF) of urine samples is then employed to provide proof of rEPO abuse [3,12]. The combination of the blood parameters with urine IEF was approved by the International Olympic Committee in 2001 [7]. This test resulted in the forfeiture of medals won by three cross-country skiers in the Salt Lake City

Table 1
Detection methods of recombinant proteins

Recombinant protein	Method	Sample type	Reference
EPO	Capillary zone electrophoresis	Physiological fluid	[13]
EPO	Isoelectric focusing (IEF) ^a	Urine	[3,7,12]
EPO	Immunoassay using monoclonal antibody	Blood	[16]
hGH	Immunoassay of isoforms	Blood	[19]
hGH	Pharmacodynamic endpoint monitoring	Blood	[19]

^a In use since Sydney 2000 Olympics.

Winter Olympics in 2002 [3]. Capillary zone electrophoresis also has the potential for detection of rEPO. This technique is based on the different glycosylation patterns between endogenous and rEPO; the main peculiarity is due to the difference in sialic acid groups [13].

Although immunoassays are of little use in direct detection of recombinant protein abuse, they play an essential role in the determination of the indirect markers of rEPO abuse. Different immunoassays can be used for determination of serum EPO and soluble transferrin receptor concentrations [14,15]. Potential for a greater role of immunoassays in the detection process is generated by the production of a monoclonal antibody against rEPO which has an affinity constant of 5.0×10^5 L/mol against the coated antigen [16].

Sample integrity issues may pose additional complications to detection of recombinant proteins. For example, there is a need to protect EPO against proteases in urine samples used in IEF [17]. Another problem is presented by the time and temperature dependence of blood samples to be analyzed for rEPO abuse. Such samples have to be analyzed as soon as possible, preferably on-site of competition, to avoid decline of red blood cell analytes with time and temperature [18]. Reticulocyte percentage and hematocrit level (reliable only for a few hours) were found to be the most vulnerable [18]. Sample transportation to other testing locations even at low temperatures would pose a significant risk of sample analyte deterioration due to mechanical stress. Another important issue to be considered is the effect of hematologic abnormalities in the tested athletes, which are unrelated to the doping process. However, it was found that most hematologic disorders found in top athletes do not lead to values higher than the cutoffs for the rEPO model tests; actually, most were found to give lower values. Therefore, the effect of such conditions on rEPO testing is mostly negligible [9].

Human growth hormone (hGH)

The inherent features of hGH made the detection of rhGH an even more troublesome endeavor than that of rEPO. hGH has a short half life (~15 min) and exists in urine samples at very low concentrations. As a result, urine samples could not be used for testing. One approach is based on the use of immunoassays for detection of different isoforms of endogenous hGH [19]. rhGH consists only of one isoform which is 22 kDa, whereas the endogenous hGH consists of different isoforms with various sizes. Administration of rhGH (22 kDa) will repress pituitary secretion of hGH by negative feedback. Thus, if immunoassay analysis of serum shows abnormally elevated levels of the 22 kDa protein, this would indicate illegal use of rhGH [19].

Another approach avoids detection of hGH protein itself, instead, it focuses on the pharmacodynamic endpoints of hGH action, i.e., changes in parameters modulated by hGH. hGH exerts most of its functions through generation of insulin-like growth factor (IGF-1). Treatment of athletes ($n = 15$) with 0.06 IU hGH/kg/day for 14 days resulted in a rapid increase in IGF-1 concentrations even 3 days after initiation of treatment [20]. It has been suggested that hGH

abusers may take up to 25 IU/day which is much higher than the dose of 1–2 IU/day given in GH-deficient patients [19]. Both hGH and its mediator, IGF-1, are potent mitogenic and anti-apoptotic agents, and several reports have shown an association between IGF-1 levels and the incidence of breast, prostate, and colorectal cancers [19,21].

The next level: gene doping

The field of gene therapy is evolving and may soon open the door for a more insidious doping method termed “gene doping”. Gene doping aims to produce recombinant proteins within human cells rather than introducing the recombinant product into the body. In the 2005 prohibited list published by the World Anti-Doping Agency (WADA), gene doping is defined as the “non-therapeutic use of cells, genes, genetic elements, or modulation of gene expression, having the capacity to enhance athletic performance” [2].

Methods of gene doping

Gene doping stemmed out of the legitimate gene therapy experiments, which are based on strategies for treating genetic diseases by introducing and expressing a deficient gene or by modulating the activity of an existing gene [3,4]. In vivo introduction of the target (artificial) gene into the human genome can be achieved by biological (viral vectors), physical (direct injection using a syringe or gene gun), or chemical methods (using phospholipids vesicles known as liposomes). Ex vivo gene doping can include gene transfer to cells in culture and re-introduction of the genetically modified cells into the host. Gene therapy or doping using viral vectors is the most efficient method in which replication-deficient vectors derived from retroviruses, adenoviruses, or lentiviruses are used to deliver the gene of interest. The genetically engineered viruses are then introduced into the body where they infect the cells and recruit the cells’ biochemical machinery to express the transgene. Such vectors offer several advantages of long-term expression, low anti-vector immunity, cell-specific tropism, and large packaging capacity [22]. However, it should be noted that integrating gene transfer vectors pose a risk of insertional mutagenesis [22].

Candidates of gene doping

Erythropoietin (EPO), human growth hormone (hGH), insulin-like growth factor-1 (IGF-1), peroxisome proliferator-activated receptor-delta (PPAR δ), and myostatin inhibitor genes have been identified as primary targets for doping [1,3,4]. Genes encoding for analgesic peptides such as endorphins and enkephalins may be used as alternatives to the widely used and panned analgesics. The biochemical, physiological, and expected performance enhancement properties of selected proteins expressed by genes targeted for doping are summarized in Table 2.

A child was born in Berlin with a mutation that turns off the myostatin gene; such a gene is believed to block the activation

Table 2
Top genes targeted for doping

Gene	Biochemical properties of expressed protein	Physiological function of expressed protein	Expected performance enhancement	References
Erythropoietin (EPO)	Glycoprotein hormone	Increases the number of red blood cells and thus increases blood oxygenation	Endurance	[3]
Insulin-like growth factor-1 (IGF-1)	Protein consisting of 70 amino acids	Increases muscle size and mass	Strength	[3]
Human growth hormone (hGH)	Protein consisting of 191 amino acids. It has a number of isoforms, the major ones have sizes of 22 kDa and 20 kDa	Increases muscle size and mass	Strength	[3]
Peroxisome proliferator-activated receptor-delta (PPAR δ)	Nuclear hormone receptor protein	Associated with the formation of type I or slow-twitch skeletal muscle and can induce their conversion from type II or fast-twitch fibers, which are determinants of endurance and speed, respectively. Furthermore, it is very likely to have a role in body weight control	Speed and Endurance	[27,28]
Myostatin	A protein consisting of two subunits, 110 amino acids each	A negative muscle mass regulator. Its inhibition would lead to limited restriction of muscle growth	Strength	[29]

of muscle stem cells in animals [23]. At 4.5 years, this boy is reported to have the physique of a mini-bodybuilder [23]. For therapeutic purposes, this mutation could be investigated to treat muscle wasting diseases. For doping, athletes can be injected with a viral vector carrying a gene that blocks myostatin. Such treatment would elicit muscle development but would be very difficult to trace.

Recently, a family of proteins termed hypoxia inducible factors (HIFs) has been identified which may contribute to

genetic manipulation for both therapeutic as well as doping purposes. HIFs are transcription factors that are naturally released under hypoxic conditions and alter the transcription of genes that affect aerobic performance such as genes encoding for EPO and glycolytic enzymes [24]. The stimulation of HIFs release under normal oxygen supply, e.g. by chemical agents, would automatically lead to drastic improvement in athletic endurance. Of note, HIFs also stimulate genes that encode molecules involved in cell growth and division and may ultimately promote cancer [24]. The ability of HIFs to modulate EPO gene expression makes their use fall under the scope of the WADA's definition of gene doping. They may also be categorized within substance doping since releasing factors for EPO are explicitly prohibited in the substance doping list [2]. The discovery of HIFs and their doping potential places an extra burden on clinical scientists to also track any drugs that may stimulate their release.

Is there an urgent need to detect gene doping?

The fact that detection of gene doping represents a top priority for sport organizations is justified by a number of factors: (1) potential to tremendously enhance athletic performance, (2) successful gene doping of animals, and (3) potential health risks. The extreme appeal of gene doping as the ultimate performance enhancement method so far is practically undisputed. The documented effectiveness of gene doping is evident by several animal trials (Table 3). It is expected that successful human trials may be only a few years away. Despite all setbacks of gene therapy, clinical trials are continuing, and new gene delivery vectors, including those based on chromosomal elements, are being developed constantly. It is expected that athletes may attempt gene doping without necessarily awaiting full approval of gene delivery trials for therapeutic purposes. The expected health side effects include lethal immune response among other complications. Opposite to gene therapy that is carried out under strict conditions and precautions, gene doping is likely to be done in secret with limited protective actions and consequently more expected health hazards. Preparation of gene transfer vectors in non-controlled laboratories may lead to their contamination with chemicals and pyrogens. Virulent viral vectors may be generated inadvertently, and these could be harmful to the athletes and the general population.

Health risks may also result from gene overexpression, a common problem in gene therapy and doping, which varies according to the type of introduced gene. Gene doping with IGF-1 or myostatin can lead to large muscles that may overload tendons and bones or cause damaging differential stresses on them. Gene overexpression may cause toxicity due to accumulation of expressed proteins and/or unusual adaptation of the body to the excess amount. Mutations also contribute to health risks due to possible integration of the genetic elements of the viral vector into the host genome and the consequent shifting or disruption of host genetic sequences [4]. Because hGH is a potent mitogenic and anti-apoptotic agent, overexpression of hGH may be related to development

Table 3
Animal studies of gene doping

Target gene	Study	Animal model	Results/side effects	Reference
EPO	Gene insertion	Macaques	Successful expression of fully functional EPO protein and a significant increase in red blood cells production <i>Side effects:</i> High increase in blood viscosity hampering normal blood flow and heart functioning Severe immune response towards endogenous and new EPO	[3,25] [30]
EPO	Gene insertion using an adenovirus	Mice and monkeys	Increase in hematocrit by 49–81% (mice) and 40–70% (monkeys) Effects lasted for 12 weeks in monkeys and more than a year in mice	[26,31]
PPAR δ	Gene insertion	Mice	Running time improved by 67%, while the distance improved by 92% Resistance to obesity even in lack of exercise and on fat-rich diet	[28]
IGF-1	Gene insertion using an adeno-associated viral vector	Mice	20–30% increase in muscle strength and mass and an increase in endurance	[3,25]

of tumors [19]. Unfortunately, this extensive list of hazards is not expected to deter dopers because the rewards of athletic excellence: financial, social, and psychological, are tempting enough for many athletes to willingly take the risk.

Possible strategies for detecting gene doping

A number of conceptual and practical factors have led to the fact that no tests are currently available to detect gene doping [3,4]. In addition to the fact that the protein produced by the foreign gene or genetically manipulated cells will be identical to the endogenous one, most gene doping proteins, particularly muscle enhancing ones, are generated locally in the muscle and do not show in blood or urine, as is the case with IGF-1. The only reliable assay would require a muscle biopsy; such an assay is virtually inapplicable in a sports setting. Nevertheless, a number of other promising strategies are currently being investigated.

Structural difference of proteins encoded by transgenes

Slight structural differences between native and expressed proteins may be used as basis for detection. Monkeys doped by the EPO gene at the French National Doping Laboratories produced EPO that has a slight structural difference from the endogenous one [3,25]. Although the exact cause of this difference is not fully understood, there is a possibility that proteins in different cells do not undergo the same post-translational modifications; this gives new hope for detection. The difference would account for a different immune response by the host that could be traced. Furthermore, this observation raises strong suspicion that upon doping different tissues produce different EPO [25]. This makes EPO, a top doping transgene candidate, another step closer to detection.

Immune response to viral vectors

Viral vectors are the most popular methods for gene delivery and are most likely to be used for gene doping. Direct detection of gene doping vectors is difficult as they are only measurable shortly after administration and may require tissue sampling. However, test strategies based on assessing the host's immune response to viral vectors may prove rather effective. Nevertheless, on the downside of this approach, there is a possibility that the tested athlete could have been infected by the virus via non-doping routes, and therefore the detection of antibodies in his/her blood will not provide conclusive evidence of doping. Furthermore, it is quite possible to genetically engineer the viral vectors to minimize any immune response.

DNA microarrays and proteomic profiling

DNA microarrays allow for another promising testing approach by providing the ability to assess the expression profile of endogenous genes that may be altered following the expression of a foreign gene. It may be also possible to monitor gene doping by observing changes in protein profiles by performing multiple proteomic profiling of athletes. Developing a reference database for different genetic and proteomic profiles that change following doping as well as establishing corresponding normal values would be the major difficulty facing this strategy.

DNA bar codes

DNA bar-coding has been proposed to provide a way to track agricultural products generated by genetic manipulations. A short stretch of genetic code unique to each transgene, to distinguish it from other transgenes and its endogenous counterpart, could be added to transgenes and viral vectors. Similar to supermarket barcodes that are used to identify different products, the DNA barcode can be used to identify transgenes and their manufacturing companies [26]. A simple molecular diagnostic test, e.g. PCR, can be employed to detect the presence of the transgene. Constructing a database of

barcodes would facilitate tracking transgenes. Such an initiative would greatly boost the detection efforts, but at the same time, it would require immense coordination among various organizational bodies. These include national and international sports federations and organizations, athletes, ethicists, and the involved diagnostic and pharmaceutical corporations. The latter may pose the biggest obstacle since they are likely to oppose the idea in fear of extra cost and jeopardizing commercial secrets and advantages.

Is good science enough?

Technical challenges are not the only obstacles facing detection efforts. There are cost-related issues that could become a major limitation to anti-doping efforts. Doping prevention efforts, from basic research and development of new diagnostic strategies to the awareness and coordination of programs, are extremely costly. The fight against doping also involves huge educational, awareness, and monitoring projects, which fall within the WADA's scope of activities.

The WADA has contingency plans in place for emerging issues such as doping, however. According to its 2004 annual report, the WADA has dedicated \$14 M for research projects between 2001 and 2004 [2]. The four main research priorities of WADA are: (1) compounds and/or methods enhancing oxygen delivery, (2) compounds and/or methods enhancing growth, (3) gene and cellular technologies applied to doping, and (4) miscellaneous projects relating to the List of Prohibited Substances. Clearly, research involving detection of recombinant proteins and gene doping constitutes at least three of the WADA's four priority research themes [2].

The total number of samples analyzed by the 32 accredited laboratories in both Olympic and non-Olympic sports increased by almost 12% between 2003 and 2004 [2]. The increasing threat of gene doping and recombinant proteins can be expected to dramatically impact the sample volume to be tested at major sports events. The capacity, speed, and technologies of anti-doping laboratories are improving, but so are the numbers of Olympians and the demand for stricter doping control in competitive sports. The most difficult challenge may be the logistics involved in coordinating the various international authorities and athletes for education about doping. These challenges extend to corporations involved in producing recombinant proteins. It is difficult to accurately estimate the total cost of doping control; however, this complicated issue must be navigated carefully because it extends far beyond scientific excellence and athletic dedication. In any case, WADA appears to have little choice except to take an aggressive and comprehensive stance because the integrity of sporting competition is at stake.

What lies ahead?

As the recombinant DNA technology advances, the potential of doping methods increases. The quality of recombinant proteins used is likely to improve, and their detection to become even more challenging. At the same

time, the appeal of the doping method and the increasingly lucrative nature of competitive sport make the situation even more difficult. Despite the fact that gene therapy trials have been hampered by the death of one volunteer and the development of leukemia in others, some athletes are willing to take risks, and gene doping is expected to be seen in practice as soon as the Beijing 2008 Olympic Games. This puts increasing pressure on clinical scientists to develop reliable and practical methods for its detection, alongside keeping up with the current notorious substance doping using recombinant proteins. Although the concepts of efficient detection are practically fully developed, the actual challenge lies in resolving the technical aspects of the detection methods. These include pre-analytical variables such as vigorous exercise, storage conditions, and time elapsed for samples to reach the anti-doping laboratories.

It should be noted that most athletes may not have enough background to fully comprehend the potential risks imposed by gene doping. Therefore, education of athletes as well as their supporting staff would be crucial in minimizing both the abuse of recombinant proteins and gene doping. One can sum up the current challenge into the following: how can one practically differentiate among substances that have the same structure, do the same job in the same place? This is the exact question sports clinical chemists are trying to answer at the moment.

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