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REVIEW

Erythropoiesis-stimulating agents and other methods to enhance oxygen transport

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Oxygen is essential for life, and the body has developed an exquisite method to collect oxygen in the lungs and transport it to the tissues. Hb contained within red blood cells (RBCs), is the key oxygen-carrying component in blood, and levels of RBCs are tightly controlled according to demand for oxygen. The availability of oxygen plays a critical role in athletic performance, and agents that enhance oxygen delivery to tissues increase aerobic power. Early methods to increase oxygen delivery included training at altitude, and later, transfusion of packed RBCs. A breakthrough in understanding how RBC formation is controlled included the discovery of erythropoietin (Epo) and cloning of the *EPO* gene. Cloning of the *EPO* gene was followed by commercial development of recombinant human Epo (rHuEpo). Legitimate use of this and other agents that affect oxygen delivery is important in the treatment of anaemia (low Hb levels) in patients with chronic kidney disease or in cancer patients with chemotherapy-induced anaemia. However, competitive sports was affected by illicit use of rHuEpo to enhance performance. Testing methods for these agents resulted in a cat-and-mouse game, with testing labs attempting to detect the use of a drug or blood product to improve athletic performance (doping) and certain athletes developing methods to use the agents without being detected. This article examines the current methods to enhance aerobic performance and the methods to detect illicit use.
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Abbreviations: 2,3-DPG, 2,3-diphospho glycerate; *EPO*; Epo, erythropoietin (gene; protein); eEpo, endogenous Epo; ESA, erythropoiesis-stimulating agent; FOB, follow-on biologic; HIF, hypoxia-inducible factor; HIF-PH, HIF-prolyl hydroxylase; IEF, isoelectric focusing; O_2, oxygen; Peg-epoetin beta, polyethylene glycol-conjugated epoetin beta; rHuEpo, recombinant human erythropoietin; RBC, red blood cell; sTfR, soluble transferrin receptor; VO_{2max}, maximum capacity to transport and utilize oxygen

Introduction

A primary function of Hb residing within red blood cells (RBCs) is to bind oxygen (O_2) under conditions of high O_2 concentration (in lungs) and transport and release it to tissues where O_2 is being consumed (for example, muscles and brain). RBCs are the most abundant of total circulating cells, representing approximately 40–45% of the total blood volume and 99% of all circulating cells. Hb constitutes 99% of the cytosolic protein in mature RBCs (Hebbel and Eaton, 1989). This large amount of Hb is consistent with the requirement for a large O_2 transport capacity that must support the considerable consumption of O_2 in tissues. As O_2 consumption increases dramatically with exercise, adjustments in O_2-carrying capacity are made to meet the increased demand.

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Erythropoietin (Epo) is a circulating glycosylated protein hormone that is the primary regulator of RBC formation. Endogenous Epo (eEpo) is produced in amounts that correspond to the concentration of O_2 in the blood and is synthesized primarily in the kidney, although it is also made at lower levels in other tissues such as liver and brain (Koury *et al.*, 1988, 1989, 1991; Lacombe *et al.*, 1988; Maxwell *et al.*, 1993). Successful cloning of the human *EPO* gene (Lin *et al.*, 1985) allowed for production of recombinant human erythropoietin (rHuEpo), and later the approval to treat patients with anaemia (low Hb levels) in humans. This breakthrough allowed many patients for the first time to resume their normal daily activities due to increased energy.

Unfortunately, some athletes and their coaches were eager to abuse rHuEpo because it increases the O_2 supply to the muscles and boosts performance in endurance sports such as skiing, running and cycling. This led to a view among some athletes that to compete successfully doping with rHuEpo was required, forgetting that inappropriate use was associated with increased risk to the athlete as well as to the sport itself.

This review will explore the ergogenic benefits associated with enhanced O_2 delivery to tissues, and the methods, risks and detection strategies for agents used in doping. For additional discussions, the reader is directed to several excellent reviews on the subject (Kazlauskas *et al*., 2002; Catlin *et al*., 2003; Gaudard *et al*., 2003).

Erythropoiesis and erythropoietin

Erythropoiesis is the process whereby erythroid precursor cells proliferate and differentiate into RBCs (Figure 1). Early haematopoietic progenitor cells residing primarily in the bone marrow differentiate into burst-forming unit erythroid cells, so named because of the characteristic 'burst' colonies formed in cell culture in semisolid medium. These cells will further differentiate into colony-forming unit erythroid cells, which acquire responsiveness to eEpo. Further stimulation of colony-forming unit erythroid with eEpo results in synthesis of Hb and differentiation into proerythroblasts, and finally, erythroblasts. These erythroblasts will enucleate, resulting in reticulocytes that are so named because of the 'reticulin' associated with the presence of RNA. After several days, reticulin declines and the cells become mature RBCs. Under normal conditions in humans, RBCs have a prolonged lifespan (3–4 months) (Smith, 1995).

A primary role of Hb in RBCs is to carry O_2 to O_2-dependent tissues. Hb found in RBCs is a tetrameric haem iron-containing protein. In adults, Hb is predominantly comprised of two α-subunits and two β-subunits. Tetrameric Hb is an important carrier of O_2 from the lungs where O_2 concentration is high (for example, lungs) to tissues where O_2 concentration may be low (for example, muscles). The affinity of O_2 for Hb in RBCs is increased with low temperature, low CO_2 and low 2,3-DPG (2,3-diphospho glycerate) as occurs in lungs. Affinity is reduced by increases in body temperature, hydrogen ion, 2,3-diphosphoglycerate or carbon dioxide concentration (the Bohr effect) under conditions where O_2 levels are low, such as working muscles. Consequently, in muscles that are consuming O_2 and generating CO_2 and lactic acid, O_2 is released from Hb.

Erythropoiesis is stimulated by eEpo, and under conditions of severe hypoxia (low O_2 concentration) eEpo levels can increase up to 1000-fold (Erslev, 1997). Epo is initially synthesized as a 193 amino-acid precursor. During transit through the secretory apparatus, the 27 amino-acid signal peptide and C-terminal arginine are removed, and carbohydrate chains are added to three N-linked glycosylation sites and the one O-linked glycosylation site. The secreted protein contains 165 amino acids with approximately 40% of the mass composed of carbohydrate (Browne *et al*., 1986).

Recombinant human erythropoietin: benefits and risks in the treatment of anaemia

Recombinant human erythropoietin is an erythropoiesis-stimulating agent (ESA) that, when first introduced as Epoetin alfa, was a breakthrough therapeutic for anaemia associated with kidney disease, chemotherapy in cancer, HIV and blood loss following surgery or trauma. The clinical benefits of rHuEpo in these indications are well understood and appreciated. A number of studies have demonstrated that rHuEpo is well tolerated and effective at raising Hb levels in end-stage renal disease patients receiving haemodialyses (Eschbach *et al*., 1989) and in patients with chemotherapy-induced anaemia (Glaspy, 2003). Treating anaemia with rHuEpo reduces the likelihood of blood transfusions, restores energy levels (Guthrie *et al*., 1993), increases physical exercise tolerance due to increased O_2 delivery to muscles and brain (Horina *et al*., 1991; Davenport, 1993; Fagher *et al*., 1994). It also improves cognitive function, the sense of well being (Grimm *et al*., 1990; Horina *et al*., 1991; Pickett *et al*., 1999) and improves patient quality of life (Delano, 1989; Deniston *et al*., 1990; Harris *et al*., 1991; Moreno *et al*., 1996).

Current medical practice involving RBC transfusion or administration of ESAs as anaemia therapy is typically

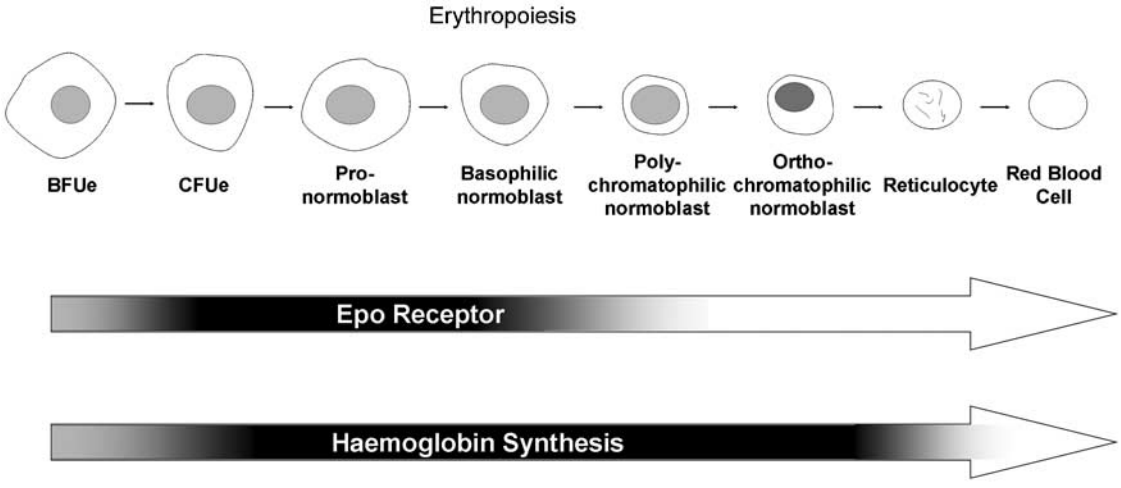

Figure 1 Erythropoiesis. Early erythroid progenitor cells proliferate and differentiate into mature Hb containing red blood cells (RBCs). RBCs lack a nucleus and have only a limited capacity to synthesize proteins, such as Epo receptors, which are absent from these cells. Darkened sections of the arrows indicate times of Epo receptor expression and Hb synthesis. BFUe, burst-forming unit erythroid; CFUe, colony-forming unit erythroid.

limited to certain patient populations. The ESA dose, ESA dose frequency, rate of rise of Hb, as well as target Hb levels are carefully monitored and controlled to maximize benefit while minimizing the possible risk associated with higher Hb concentrations. The target Hb in such patients (generally a value or range between 10 and 12 g per 100 mL depending on the indication, physician prescribing information or clinical guidelines followed) is typically below that found in normal individuals (13–15 g per 100 mL). Adverse events associated with ESAs are multifactorial in origin. Anaemic patients with cancer who are treated with ESAs show evidence of increased frequency of thrombotic events (Bohlius *et al.*, 2006). Severe elevation in haematocrit (Hb≅haematocrit divided by 3) in haemodialysis patients treated with ESAs is associated with hypertension, thromboembolism and decreased survival (Regidor *et al.*, 2006).

Patients and athletes who dope with ESAs may also be administered i.v. or oral iron to ensure that synthesis of Hb, and, consequently, erythropoiesis is not impaired due to insufficient iron levels. Although i.v. iron when used appropriately is considered to be safe, excessive use can lead to serious complications including increased oxidative tissue damage and increased cardiovascular risk (Kalantar-Zadeh *et al.*, 2005).

Pharmaceutical companies, regulatory agencies and the medical community have carefully examined the benefit–risk ratio and have developed guidelines for appropriate manufacture, distribution and use of ESAs and iron (KDOQI and National Kidney Foundation, 2006; Rodgers, 2006).

Risks of ESA use in healthy individuals

Although the adverse events associated with ESA use in patients with disease may not apply directly to healthy athletes, prudence suggests that the excessive use of ESAs should be avoided. In contrast to that of patients with anaemia, athletes may administer rHuEpo or transfuse RBCs to elevate their Hb levels above the normal range. Elevated haematocrit and dehydration associated with intense exercise may reveal undetected cardiovascular risk in some athletes. The risk of elevated haematocrit/Hb levels has been described in humans with polycythaemia (Spivak, 2002). Subjects with Chuvash polycythaemia have elevated eEpo levels and polycythaemia. These subjects have an altered response to O_2 due to a mutation in VHL, a protein important in the regulation of the transcription factor that controls synthesis of several genes including eEpo (Ang *et al.*, 2002). Affected individuals develop peripheral vein varicosities, hypertension and increased vascular complications, including thrombotic events and a shorter lifespan (Gordeuk and Prchal, 2006). In some studies with mice, overexpression of Epo can lead to polycythaemia and premature mortality (Semenza *et al.*, 1989; Villeval *et al.*, 1992; Prchal *et al.*, 1995; Wagner *et al.*, 2001). Increased blood pressure was observed in rats treated with rHuEpo (Vaziri *et al.*, 1995). The association between polycythaemia and survival is not completely understood because other groups found no shortening of lifespan in a series of transgenic mouse lines overexpressing human Epo protein with elevated mean haematocrits ranging from 48 to 80% (Madan *et al.*, 1995; Kochling *et al.*, 1998). Similarly in humans with primary familial and congenital polycythaemia, the condition is thought to be relatively benign, the disease is non-progressive and the affected subjects have a normal lifespan. However, some individuals are reported to have died from stroke, have hypertension or suffer from recurrent thrombotic complications (Prchal and Sokol, 1996).

Other rarely discussed risks of doping include those associated with inappropriate storage and/or mishandling of the drug, a possibility linked with illicit use. For example, products that are transported in the back of the car or via other inappropriate storage options where elevated temperatures frequently occur can result in product degradation, which may affect not only product quality but also safety. Inappropriate storage of ESAs may result in the generation of immunogenic degradation products, such as aggregates, which are thought to play an important role in immune reactions to protein therapeutics (Smalling *et al.*, 2004). Immune reactions to rHuEpo can lead to antibody-mediated pure red cell aplasia, a serious condition where antibodies to the product cross-react with the endogenous hormone, thereby inactivating it. The result is severe anaemia due to a deficit in levels of active circulating eEpo (Casadevall, 2003; Smalling *et al.*, 2004).

In addition to the harm that can affect the user, pharmaceutical companies are troubled by doping with their medicines for a number of reasons. These include the lack of rational safety assessment associated with inappropriate use. Occasionally, drugs are used before safety tests have been completed, and athletes are taking great risks because the safety issues are not completely understood. The athlete who abuses medicines considers only the benefit to performance and ignores the potential short- and long-term liabilities. Such inappropriate use bypasses the well-established partnership between drug companies and regulatory agencies, who wish to maximize the benefit–risk ratio.

Doping in athletes

Accepted methods to increase Hb levels and performance included diet, vigorous training or training at altitude. The benefits of altitude training were understood, however, such training was not without risk or side effects (Maggiorini, 2006; Rupert and Koehle, 2006). Some individuals can cope with the excessive erythrocytosis associated with high altitude (Mejia *et al.*, 2005), as can some subjects with idiopathic polycythaemia (Finazzi *et al.*, 2006), because of adaptation by increasing heart rate, ventilation, vasodilation and reduced viscosity allowing them to avoid the most severe effects of altitude sickness. However, some do not and can suffer from serious complications. Thus, careful adaptation to high altitude is warranted (Vardy and Judge, 2005).

Athletic performance enhancement, or 'doping', by administration of autologous or homologous blood was reported as early as the 1970s (Ekblom, 2000). The traditional methods such as altitude training alone did not show the same magnitude of increase in Hb levels as obtained by RBC transfusions (Ashenden *et al.*, 2001) explaining its use. However, the complexity, time required, storage issues and risks associated with RBC transfusion(s) made this method undesirable.

The search for other agents and methods that could provide ergogenic benefit in athletes continued. The successful cloning of the *EPO* gene (Lin *et al.*, 1985) was followed quickly by clinical trials, and clinical use of the first ESA, Epoetin alfa. Clinical use of Epoetin alfa in humans began in 1988 in Europe and in 1989 in the United States. The performance benefits of rHuEpo were noted and inappropriate use by some athletes began (Catlin *et al.*, 2003).

Anecdotal stories suggested doping with rHuEpo began as early as the Calgary Winter Games in 1988, followed by rumours of deaths of Dutch cyclists associated with inappropriate use of rHuEpo in 1989 (Catlin *et al.*, 2003). An analysis of Hb levels of athletes competing at the world cross-country ski championships in 2001 compared with that of the same event in 1989 showed an abnormal increase in Hb levels and reticulocytes in the top finishers (Stray-Gundersen *et al.*, 2003). Hb levels in some ski teams increased significantly in 1994 and further in 1999 (Videman *et al.*, 2000). Recently, several cyclists, Erik Zabel, former teammate Rolf Aldag and winner Bjarne Riis, have admitted to doping with rHuEpo in the 1996 Tour de France. An Epo-doping scandal surfaced with a vengeance in 1998 when French police arrested Willy Voet, a physiotherapist of the Festina cycling team, for possession of rHuEpo and other substances at the Tour de France. Following development of a direct test for rHuEpo, some urine samples from the 1998 Tour de France were reported to be positive for rHuEpo (Lasne and De Ceaurriz, 2000). Subsequently, Jascke admitted he was using rHuEpo when he finished eighteenth on the 1998 Tour. Three skiers (Johann Mühlegg competing for Spain and Olga Danilova and Larissa Lazutina competing for Russia) were disqualified during the 2002 Winter Olympic Games when darbepoetin alfa was found in their urine samples. Darbepoetin alfa is a glycosylation analogue of rHuEpo with a longer circulating half-life (Elliott *et al.*, 2003). More recently, cycling teams were plagued by doping scandals associated with the Operación Puerto doping case where bags of blood plasma were found that had high levels of rHuEpo, resulting in the withdrawal of riders in both the 2006 and 2007 Tour de France. Iban Mayo was reported to have tested positive for rHuEpo during the 2007 Tour and was suspended.

Reports of doping with rHuEpo even extended to dogs and horses to aid their performance in racing. This occurred in spite of reports that doping with rHuEpo was especially risky in animals because of the high likelihood of antibody-mediated pure red cell aplasia (Cowgill *et al.*, 1998).

The reason for doping with blood or rHuEpo was evident. There was a direct relationship between Hb levels and increased performance (Ekblom, 1996). In exercising rats, increased Hb levels resulted in increased in O_2 delivery to the brain and increased muscle fatty acids and glycogen with reduced accumulation of lactate (Lavoie *et al.*, 1998). An improvement of up to 5–10% was estimated in humans (Birkeland *et al.*, 2000; Wilber, 2002) due to increased maximum capacity to transport and utilize oxygen (VO_{2max}), velocity at VO_{2max} and maximal aerobic power (Kanstrup and Ekblom, 1984; Ekblom, 1996; Ashenden *et al.*, 2001). Each of the enhancements can translate directly into a change in time-trial performance and in long-distance events (Levine and Stray-Gundersen, 1997). The benefit from increased Hb was comparable whether the increase was due to transfusion or rHuEpo administration (Buick *et al.*, 1980; Ekblom, 1996; Birkeland *et al.*, 2000; Lippi *et al.*, 2006b), suggesting that the improvement was due to increased Hb and resulting increased O_2-carrying capacity, not the method by which Hb levels were raised.

Considerations in doping and antidoping initiatives

There are those who wish to obtain the benefits of doping, ignore risks, and circumvent the rules, test and testing procedures. The strategies to conceal doping with rHuEpo included selective timing of the rHuEpo dose and rHuEpo-dosing schedule to ensure that drug levels were below the level of detection. Athletes have also reduced risk of detection by using new agents as they became available and assumed that no test existed. Others attempted to alter samples to destroy or interfere with the test. Sports agencies have responded to use of such agents and strategies by making their use illegal and penalized those who broke the rules.

With some agents; for example, RBC transfusions, plasma volume expanders, artificial blood or O_2 carriers, the agent is present in the body during the competition. These agents expose the subject to increased risk of detection because collection of samples (urine or blood) typically coincides with the sporting event. With other agents; for example, ESAs, the effect is indirect and the agent need not be present in the body to derive benefit. This is because RBCs have a disproportionately long lifetime (3–4 months) compared with the lifetime of the ESA (hours to days) in the body. Adjusting time of dosing to the precompetition period or reduction of the doses to the minimum required for erythropoiesis stimulation (microdosing) resulted in levels that were below the threshold of detection (Ashenden *et al.*, 2006).

The view that new agents could be used because no test has been developed or that detection of some agents is not possible on theoretical grounds is misleading. To deter use of illegal substances, labs do not always reveal the existence of newer tests. eEpo and rHuEpo were mistakenly thought by some to be structurally identical and therefore no test was possible that led some athletes to believe they could escape detection. The discovery of an isoelectric focusing (IEF) method to detect doping with rHuEpo was reported in 2000. The IEF test was applied to stored urine samples and revealed the use of rHuEpo in the 1998 Tour de France several years later (Lasne and De Ceaurriz, 2000). Darbepoetin alfa was first approved for use in 2001 and rumours circulated that there was no test. However, three athletes were caught doping with darbepoetin alfa during the 2002 Winter Olympic Games. Similarly, a test was developed and implemented for the 2004 Olympic games in Athens and the 2007 Tour de France to detect homologous blood transfusion(s), subjecting the riders who cheated to increased risk of getting caught (Nelson *et al.*, 2003).

Testing strategies

Direct testing of the doping agent(s) themselves is preferred; however, many times the doping agent is cleared or

metabolized quickly making detection difficult. To overcome these difficulties, out-of-competition testing has been implemented, whereby athletes are required to be available to donate samples (urine or blood).

An alternative strategy is the detection of altered biomarkers whose changes might be unique to the administered agent. Indirect testing procedures are often attainable because the doping agent frequently has a unique or more exaggerated pharmacology than is possible by natural mechanisms. Indirect testing of biomarkers suffers from the potential for false-positive results where a natural manipulation; for example, high altitude training, might mimic the effects of exogenous rHuEpo. Some genetic abnormalities may also mimic drug administration. A cross-country skier who won several Olympic gold medals was initially accused of doping. However, it was later determined that he had an autosomal dominant mutation in his *EPO* receptor resulting in erythrocytosis and haematocrit levels up to 68% (Prchal *et al*., 1985; Juvonen *et al*., 1991) (for comparison, the normal haematocrit range is 39–45%). Considerations must be made to avoid inappropriate sanctioning of athletes who, through genetics or physiology, test positive for doping.

Agents used in doping

Numerous agents that enhance O_2 delivery to tissues have been used or abused (Figure 2). The benefit–risk ratio associated with each agent differs as do the detection methods and strategies to avoid detection. The types of agents or procedures used can be divided into several categories according to their point of intervention. These include agents that directly increase O_2 transport (transfusions with RBCs or administration of non-natural O_2 carriers), administration of ESAs that increase RBC levels (for example, rHuEpo (Epoetin alfa and its follow-on biologics (FOBs)), rHuEpo derivatives for example darbepoetin alfa or gene therapy where *EPO* genes are introduced into the body to increase Epo production), hypoxia or hypoxia mimetics that stimulate production of eEpo, and agents that alter the affinity of O_2 for Hb, thereby delivering more O_2 to muscle(s) and tissue(s) where O_2 demand is greatest. Each of these strategies will be considered.

Hypoxia and hypoxia mimetics

Hypoxia is a condition of reduced O_2 concentration in breathable air or blood. A natural response to hypoxia (which can occur during aerobic exercise or changes in altitude) is eEpo synthesis, which stimulates erythropoiesis. Thus, efforts to stimulate O_2-carrying capacity through exposure to low O_2 have been explored. The physiological benefit associated with training at elevation to increase Hb levels suffers from a reduced ability to train vigorously due to the low O_2 tension at high altitude. A high–low method of sleeping at high elevation followed by training at low elevation was found to be a better training strategy than training and sleeping at either high or low elevations alone (Levine and Stray-Gundersen, 1997; Miyazaki and Sakai, 2000; Stray-Gundersen *et al*., 2001; Robach *et al*., 2006; Schmitt *et al*., 2006). A method was developed to sleep at simulated high elevation by using low O_2 tents or rooms; whereby nitrogen is substituted for O_2, thereby decreasing the O_2 partial pressure. Although this strategy is frowned upon in some quarters, it is not currently banned. This is partly because simulated altitude is less effective at increasing reticulocytes, Hb and, more importantly, VO_{2max}, than other strategies such as rHuEpo administration, thus limiting its benefit (Ashenden *et al*., 2001).

The mechanism by which hypoxia upregulates eEpo and alters metabolism is now partially understood. Hypoxia-inducible factor (HIF) is a heterodimer comprised of α- and β-subunits and is a positive regulatory protein for *EPO* gene expression (Wang *et al*., 1995; Jiang *et al*., 1996). An O_2-dependent enzyme, (HIF-prolyl hydroxylase; HIF-PH) hydroxylates HIF at specific prolines contained in O_2 degradation domains (Bruick and McKnight, 2001; Epstein *et al*., 2001; Ivan *et al*., 2001; Masson *et al*., 2001). Hydroxylated HIF is ubiquitinylated by a ligase forming a complex with von Hippel–Lindau protein, which targets HIF for degradation by the proteosome (Ivan *et al*., 2001; Maxwell *et al*., 2001; Hon *et al*., 2002). At high O_2 tension, HIF levels are low and eEpo synthesis is halted. At low O_2 tension, HIF, which is made constitutively, accumulates due to reduced activity of HIF-PH, resulting in increased eEpo synthesis.

Compounds that act as hypoxia mimetics and thereby stimulate eEpo synthesis may have utility in treatment of

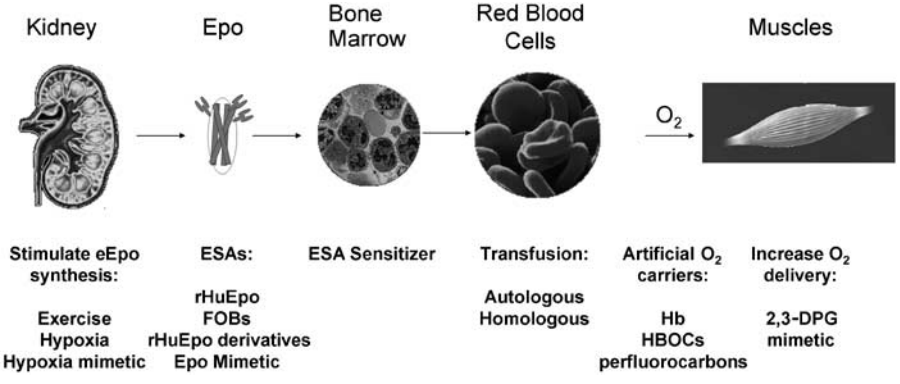

Figure 2 Methods to enhance oxygen transport include stimulation of endogenous Epo (eEpo) synthesis, stimulation of erythropoiesis (e.g., through administration of erythropoiesis-stimulating agents (ESAs) such as recombinant human Epo (rHuEpo)) or through direct increases in delivery of oxygen (e.g., transfusion, administration of artificial oxygen carriers or through enhanced oxygen unloading). 2,3-DPG, 2,3-diphospho glycerate; HBOC, Hb-based oxygen carrier.

anaemia. Several have been considered including desferrioxamine and cobalt. Desferrioxamine is an iron chelator that weakly inhibits HIF-PH (Hirsila *et al*., 2005). This agent has been used clinically to treat aluminium toxicity and iron overload (Tielemans *et al*., 1985a, b, 1988, 1989; Boyer *et al*., 1992; Davis and Porter, 2002). At high doses, it stimulates erythropoiesis activity but does so poorly. Reports of visual impairment and deaths limit its usefulness as a doping agent (Tielemans *et al*., 1985a, 1988; Roger *et al*., 1991; Hirsila *et al*., 2005). Cobalt is a potent stimulator of erythropoiesis because of its ability to stimulate production of eEpo through inhibition of HIF-PH (Katsuoka *et al*., 1983; Hirsila *et al*., 2005). Indeed, the original erythropoietic unit was defined as the erythropoietic response associated with administration of 1 mL of 5 mM cobalt to starved rats (Goldwasser and White, 1959). Early use of cobalt to treat anaemia in kidney dialysis patients, as well as cancer, and sickle cell patients was met with some success (Shen and Homberger, 1951; Wolf and Levy, 1954; Curtis *et al*., 1976).

These observations have led to a fear that cobalt administration might be used to enhance erythropoiesis in athletes (Lippi *et al*., 2006a). Although cobalt is an essential trace element, toxic effects of excessive administration have been described in humans and animals, including organ damage, impaired thyroid activity and goiter formation (Domingo, 1989; Nordberg, 1994; Barceloux, 1999). Cobalt was added to some types of beer to reduce foaming, primarily in Canada and Belgium, and heavy drinkers developed cobalt cardiomyopathy (Alexander, 1972) and subsequently cobalt addition was halted. Thus, cobalt ingestion can be associated with significant clinical sequelae.

Attempts to discover and develop more specific and safe HIF-PH inhibitors have been described (Mole *et al*., 2003). This effort has been met with some success with FG-2216 (Fibrogen Inc., South San Francisco, CA, USA), an orally active, small molecule in clinical development for the treatment of anemia which showed enhanced erythropoiesis in haemodialyses patients (Mealy and Bayes, 2005; Bunn, 2007). However, FG-2216 clinical trials were at least temporarily halted due to a drug-related liver failure and death of a patient.

Currently, there are no direct tests that have been implemented to detect doping with hypoxia mimetics, although standard methods may be sufficient. In addition, indirect tests that detect biomarkers associated with enhanced erythropoiesis such as elevated serum eEpo, Hb, reticulocytes or larger and newly formed RBCs (macrocytes) may show some promise in detecting their illicit use.

Blood and blood substitutes

Transfusion with packed RBCs has been used to treat anaemia in patients or to replace RBCs lost during surgery or an accident. Transfusions can be autologous (blood donation by and re-infusion into the same subject) or homologous (infusion of someone else's blood). The former is safer (no risk of blood type mismatch or immune reactions). Homologous transfusions have risks of blood-borne infectious diseases and the possibility of a transfusion reaction. In both situations, damaged RBCs may release free Hb resulting in sudden changes in blood pressure. Free iron or Hb can produce reactive O_2 species that can catalyse lipid oxidation, promoting atherosclerosis and increased oxidative damage to cardiovascular tissues and other organs. Repeated RBC transfusions can lead to iron overload that can then lead to serious complications such as impaired neutrophils, T-cell response and macrophage function, and more serious complications associated with iron deposition in heart, liver, pancreas or other essential organs. Elevated iron may also increase susceptibility to infection and enhance virulence of some bacterial pathogens (Kletzmayr *et al*., 2002; Puntarulo, 2005; Bullen *et al*., 2006; McCullough and Bartfay, 2007).

Autologous transfusions are currently difficult to detect, but testing labs are exploring the possibility that stored RBCs may have alterations compared with normally circulating RBCs. For example, 2,3-DPG levels are depleted in stored and re-transfused blood. In contrast, homologous blood is possible to detect because of the presence of mismatched RBC blood group antigens. It is estimated that over 20 different blood group antigens are present on RBCs (Daniels, 1999). The dominant antigens A, B, O and Rh are typically matched between donor and recipient to minimize risk of transfusion reactions; however, additional minor antigens are present and the probability is very high that homologous blood will differ from that of the athlete in at least one. A transfusion of one pint of blood will represent approximately 5–7% of the total circulating RBCs indicating that significant numbers of homologous RBCs are present. Flow cytometry is particularly sensitive and can detect rare antigenically distinct cells in mixed cell populations. A current testing method detects a panel of $\sim$10–12 blood group antigens using specific antisera and flow cytometry (Nelson *et al*., 2003). The assay can detect rare cells with immunologically distinct antigens when present at levels as low as 0.07% (Nelson *et al*., 1994). As mentioned, the lifespan of a RBC is 3–4 months (Eadie and Brown, 1953), thus transfused blood can be detected for many weeks after a transfusion.

Artificial oxygen carriers and 2,3-DPG mimetics

Artificial O_2 carriers such as Hb-based oxygen carriers (HBOCs) and perfluorocarbons (PFCs) can bind and deliver O_2 to tissues. These agents were developed as blood substitutes to improve O_2 delivery to patients with acute blood loss or an urgent demand for O_2 delivery; for example, cardiac surgery (Schubert, 2001). In solution, the Hb tetramer readily dissociates into monomers and dimers that are cleared rapidly by the kidneys. Cross-linking of Hb can prevent breakdown of tetramers into dimers and this is also thought to reduce nephrotoxicity. The short half-life of these molecules indicates that they must be infused at the time of need to derive benefit.

Perfluorocarbons are useful as O_2 carriers (Gaudard *et al*., 2003; Lippi *et al*., 2006b) because emulsions of these molecules dissolve high concentrations of O_2 that can be extracted by O_2-deprived tissues. However, unlike Hb that has a sigmoidal relationship between pO_2 and O_2 binding, PFCs have a linear one. Thus, at a given partial pressure of O_2, Hb binds significantly more O_2 than can be dissolved in the PFC.

The utility of HBOCs and PFCs as ergogenic aids is debatable because they do not respond to 2,3-DPG so are less effective at oxygenation compared with packed RBCs. HBOCs promote vasoconstriction, thereby reducing the ability to deliver O_2. PFCs require a high O_2 environment to work well as O_2 carriers and are most effective as continuous infusions, a scenario not compatible with athletic events (Lippi *et al.*, 2006b; Eichner, 2007).

Both classes of agents have considerable side effects. HBOCs can increase blood pressure (vasopressor effects attributable to scavenging of nitric oxide by cell-free Hb); decrease cardiac output; and increase or cause malaise, abdominal pain, haemoglobinuria and renal toxicity (Klein, 2005; Lippi *et al.*, 2006b). HBOCs can also increase concentrations of free O_2, which form hydrogen peroxide and peroxynitrite that were thought to be a cause of the tissue injury observed in clinical trials with these agents. RBCs avoid this problem because of cellular enzymes (catalase and superoxide dismutase) that destroy hydrogen peroxide. Although PFCs do not consume nitric oxide and thus do not have the same problems with vasoconstriction as HBOCs, they have their own side effects, including back pain, malaise and transient fever (Lippi *et al.*, 2006b).

Blood substitutes, while showing some benefit, are readily detected (Schumacher *et al.*, 2001). Synthetic Hb and HBOCs impart a red colour to serum, thereby simplifying detection. HBOCs can also be detected with routine laboratory tests including western immunoblotting (Lasne *et al.*, 2004a), or by a combination of HPLC and mass spectrometry (Simitsek *et al.*, 2007). PFC is not metabolized by the body, but as it is exhaled through the lung, it can be measured with chromatography, thermal vapour analyser or infrared absorption (Shaffer *et al.*, 1997; Mazzoni *et al.*, 1999).

Like the 2,3-DPG found in RBCs, 2,3-DPG mimetics shift the O_2 dissociation curve of Hb to the left, thereby delivering more O_2. RSR13 one such 2,3-DPG mimetic (Efaproxiral; Allos Therapeutics Inc., Westminster, CO, USA), may theoretically be used to dope because of a reported ability to increase VO_{2max} in dogs (Richardson *et al.*, 1998). Its targeted indication was to improve tissue oxygenation in patients with tumours; however, in phase 3 clinical trials, it failed to meet its primary end point and development has stopped. Its half-life is relatively short, 4–5 h, but RSR13 can be easily detected in urine within 24 h of use by gas chromatography/mass spectrometry (Breidbach and Catlin, 2001).

Erythropoiesis-stimulating agents

Recombinant human Epo. The mistaken assumption that rHuEpo is structurally 'identical' to eEpo led some to believe that a direct test to detect doping with rHuEpo was not possible. eEpo is a complex biological whose properties are affected by the cell type that produces it (interstitial fibroblast cells in the kidney and hepatocytes in the liver). The eEpo-producing cell is impacted by forces unique to the body, including a complex interaction of circulating growth factors and nutrients and particular cell–cell interactions. The secreted eEpo is subjected to differential clearance mechanisms, and all of the above can affect the physical and biophysical properties of the molecules, including three-dimensional structures of the peptide (Kung and Goldwasser, 1997) and the pattern of microheterogeneity associated with post-translational processing (Lasne and De Ceaurriz, 2000). In contrast, rHuEpo is made from non-natural transformed cells such as Chinese hamster ovary cells under controlled growth conditions, and then is subjected to purification procedures and storage conditions. These manufacturing methods do not mimic the natural process, indicating that differences between natural and rHuEpo will exist and that these differences can be detected in tests.

Both rHuEpo and eEpo have post-translational addition of three N-linked and one O-linked carbohydrate chain(s) that each can have variations in size, content of various sugars, branching pattern, chain length and composition (Sasaki *et al.*, 1987; Takeuchi and Kobata, 1991; Rush *et al.*, 1995). eEpo and rHuEpo also have a natural variation in charge due to the presence of a variable number of sialic acids (up to four) on each of the three N-linked carbohydrate chains and up to two sialic acids on the single O-linked carbohydrate chain. Thus, both eEpo and rHuEpo may have up to 14 sialic acids in total (Egrie and Browne, 2002b). Although both rHuEpo and eEpo have the same number of carbohydrate chains that are made up of the same sugars, they can differ in the linkages between sugars and the proportions of the various glycoforms. They can also differ according to charge due to differences in content of negatively charged sulphate. About 3% of the carbohydrate chains on Epoetin alfa contain sulphate and the content is typically limited to one per chain. eEpo can be extensively sulphated with as many as three per carbohydrate chain. Thus, eEpo can be considerably more negative (acidic) than rHuEpo (Strickland *et al.*, 1992; Kawasaki *et al.*, 2001). As a result, eEPO as secreted in the urine can be differentiated from rHuEPO by IEF gels.

Lasne and colleagues combined diafiltration with IEF and double immunoblotting (Lasne *et al.*, 2002; Lasne, 2003) and demonstrated that rHuEpo had a different 'fingerprint' with more basic isoforms compared with urinary eEpo (Lasne and De Ceaurriz, 2000). Some differential clearance of the most basic isoforms of rHuEpo (Egrie and Browne, 2002a) resulted in an enrichment of the more acidic species over time (Catlin *et al.*, 2002; Lasne *et al.*, 2002, 2007a; Breidbach *et al.*, 2003); however, the pattern of administered rHuEpo compared with rHuEpo excreted in urine was not substantially altered. Thus, when used appropriately, the test can detect doping with rHuEpo.

The test and the criteria for reporting a positive rHuEpo result have evolved. The initial criteria required that the intensity of the basic bands corresponding to the position of rHuEpo must represent 80% of the total intensity. Improvements in the methods to assign identity to particular bands and better computer algorithms have aided in assessing the banding patterns in immunoblots (Breidbach *et al.*, 2003; Lasne *et al.*, 2007b). In addition, the band identification criteria have become more discriminating leading to a reduction in the number of false-negative results (Bajla *et al.*, 2005; Stolc and Bajla, 2006; Lasne *et al.*, 2007b). Positive criteria now require detection of at least three consecutive bands with twofold greater intensity than those in the acidic region, thereby reducing further the possibility of false-positive results. One group reported that strenuous

exercise could result in appearance of a band that migrated in the basic region of the gels and suggested that this could result in reporting of a false-positive result (Beullens *et al.*, 2006); however, the band did not have the characteristic fingerprint of rHuEpo and the improved criteria would prevent assignment of a 'positive' test result if the band were present.

It became possible to defeat the IEF urine test by taking advantage of the rapid elimination of rHuEpo (Souillard *et al.*, 1996). Thus, the rHuEpo fingerprint was detectable in urine and was able to be differentiated from eEpo for only a limited period of time, 4–7 days (Breidbach *et al.*, 2003). Improvements in sensitivity or specificity of the IEF urine test or detection of other molecular differences between rHuEpo and eEpo may be forthcoming.

Masking agents (proteases) to defeat the Epo urine test have also reportedly been used. This became evident with the observation that there were undetectable Epo profiles in ~15% of Epo tests (Lamon *et al.*, 2007b; Thevis *et al.*, 2007). Although some samples had low levels of eEpo because of the high haematocrits, and therefore relatively high oxygen levels in blood resulting in an inhibition of eEpo synthesis, other samples may have been manipulated. Small quantities of readily available proteases, for example, trypsin papain and chymotrypsin, will degrade Epo in urine after only a short time period. Addition to a sample during the collection process could therefore destroy both eEpo and rHuEpo giving a negative result with the urine test.

Direct tests for protease activity are available and these can detect sample manipulation. Although appropriate sample storage can allow for direct testing of protease activity in the sample, proteases themselves are subject of autolysis and therefore activity can be lost over time. However, proteases have a distinct pattern of degradation of specific proteins found in urine, for example, the pattern of albumin degradation associated with each protease is unique (Lamon *et al.*, 2007b). Therefore, the effects of protease addition are readily detected and the particular protease added can be determined. An alternative strategy is to add protease inhibitors to the collection vessel to prevent proteolysis.

Indirect methods for detection of rHuEpo abuse have also been developed. Although not proof of rHuEpo doping, testing for haematocrit or Hb levels above a certain value has been explored. A 'health test' has been implemented in some competitive sports, whereby athletes with high haematocrit or Hb levels were assumed to be at risk of adverse events and were not permitted to compete. When levels declined to a threshold deemed safe, they could resume the competition. When athletes failed the health test, presumably because of illegal means, a few either waited for their Hb levels to drop or subjected themselves to phlebotomy to reduce levels to 'legal limits'. The Hb limits have been changed over time and differ according to the sports organization, but the thresholds whereby the athlete was unable to compete are typically high (for example, International Cycling Union had haematocrit limits of 50% for men and 47% for women). Analysis of haematocrit/Hb levels in elite athletes before rHuEpo was commercially available revealed that 10% had haematocrit levels above 50% (Hb ~16.7 g per 100 mL) suggesting that some individuals may naturally exceed the limits (Schumacher *et al.*, 2000). However, Hb levels have generally declined in competitors following implementation of the health test, indicating that it met its primary objective—to deter doping (Videman *et al.*, 2000).

Recombinant human erythropoietin abuse results in changes in other biomarkers besides Hb level, including decreased eEpo level and increased numbers of young erythroid cell types such as reticulocytes and RBC macrocytes (Parisotto *et al.*, 2000, 2001). The increased erythropoietic demand due to rHuEpo stimulation results in decreased total circulating iron, ferritin and increased soluble transferrin receptor (sTfR) (Birkeland *et al.*, 2000; Parisotto *et al.*, 2000). Withdrawal of erythropoietic stimulation, particularly when Hb levels are high, results in an unusually low eEpo level, a reduced number of reticulocytes and RBC macrocytes, and increases in ferritin and total circulating iron. These changes, even though not unique to rHuEpo addition or withdrawal, are usually of a lesser magnitude than the changes associated with other mechanisms (for example, high altitude training) (Ashenden, 2002).

An indirect test measuring a combination of biomarkers associated with rHuEpo addition or cessation has been used to detect possible doping (Parisotto *et al.*, 2000; Ashenden *et al.*, 2001). Different measures were developed to detect concurrent administration of rHuEpo ('on-score') or recent cessation ('off-score'), based on equations involving various biomarkers. Parameters are 'weighted' to get contributions from multiple variables. On-scores can measure haematocrit and Epo (HE; $\mathrm{Hb} + 9.74\ln(\mathrm{Epo})$) or Hb, Epo and sTfR (HES; $\mathrm{Hb} + 6.62\ln(\mathrm{Epo}) + 19.4\ln(\mathrm{sTfR})$. Off-scores can measure Hb and reticulocytes (HR; $\mathrm{Hb} - 60\sqrt{(\mathrm{ret})}$) or Hb, reticulocytes, and eEpo (HRE; $\mathrm{Hb} - 50\sqrt{(\mathrm{ret})} - 7\ln(\mathrm{Epo})$) (Parisotto *et al.*, 2000, 2001). Typically, the criteria are set high to minimize the likelihood of false-positive results. A normal on-score is 85–95 and scores over 133 are considered evidence of doping. The first use of the test was at the 2000 Sydney Summer Olympics. It subsequently has been modified and improved somewhat to increase its efficiency (Sharpe *et al.*, 2006).

In an additional attempt to deter doping based on indirect markers of erythropoiesis, a 'haematological passport' has been implemented in some sports. Within-subject variations in certain haematological markers, for example, Hb, haematocrit, reticulocytes and sTfR are monitored continuously. Typically five or more determinations could define subject-specific reference ranges. (Malcovati *et al.*, 2003; Morkeberg *et al.*, 2007). Sudden changes in any of these parameters, for example, a 10% increase in haematocrit, would be evidence of unapproved activity and the athlete could be barred from competition. This strategy can detect doping according to multiple methods including transfusion. However, even this approach is not without critics who point out the possibility of false-positive results, thereby unfairly preventing clean athletes from competing (Lippi *et al.*, 2006c).

Darbepoetin alfa. Darbepoetin alfa is a rHuEpo glycosylation analogue that was approved for use in 2002–2003 in the European Union and United States. Compared with rHuEpo, darbepoetin alfa has five amino-acid substitutions resulting in the creation of two additional N-linked carbohydrate attachment sites. It has the same mechanism of action as

rHuEpo, binding and activation of the Epo receptor, but it has a longer serum half-life. The increased serum half-life results in increased *in vivo* potency because of the extended time the agent can stimulate erythropoiesis. It can also be differentiated from both rHuEpo and eEpo according to sialic acid content (up to eight additional negative charges), and mass (rHuEpo 30.4 kDa, darbepoetin alfa 40 kDa). The urine one-dimensional IEF test originally developed to detect rHuEpo was found to easily distinguish darbepoetin alfa from both rHuEpo and eEpo due to minimal overlap of isoforms (Catlin *et al.*, 2002; Breidbach *et al.*, 2003; Lamon *et al.*, 2007a). This test was applied in the 2002 Winter Games in Salt Lake, where three athletes were caught with darbepoetin alfa in their urine samples and they were sanctioned (Rollins, 2003). The reasons the athletes were caught during the competition was likely because of the recent marketing approval of darbepoetin alfa and the false assumption that there was no test available. In addition, the longer serum half-life increased the time the agent was in the system, thus increasing the detection window (Lamon *et al.*, 2007a; Morkeberg *et al.*, 2007) Subsequently, darbepoetin alfa has been used only rarely by athletes wishing to dope.

Follow-on biologicals and other ESAs. Although rHuEpo has a defined amino-acid sequence, differences in its production result in subtle changes in post-translational modifications including glycosylation, conformation and impurities. Currently, it is not possible for another manufacturer to duplicate exactly the product profile of the innovator. Thus, the term 'generic' is not used to describe rHuEpo molecules made by different manufacturers. Instead, the descriptors 'follow-on biologics' or FOBs, 'generic biosimilars' or 'generic biopharmaceuticals' are used (Cleland *et al.*, 1993).

The first commercial rHuEpo product introduced in the United States and European Union was Epoetin alfa and tests were developed to detect this molecule. However, appearance of FOBs of rHuEpo in the marketplace has impacted the strategies to detect abuse with these ESAs.

As explained earlier, rHuEpo microheterogeneity is in part a consequence of cell line and manufacturing conditions. Thus, the microheterogeneity and 'fingerprint' associated with the first rHuEpo, Epoetin alfa, is not identical to subsequent rHuEpo molecules made by different manufacturing processes (Yuen *et al.*, 2003; Combe *et al.*, 2005; Schellekens, 2005). The second rHuEpo approved for commercial use, Epoetin beta, was sufficiently similar to Epoetin alfa so that the original positive test criteria were applicable for both epoetins, although the banding pattern is slightly different (Storring *et al.*, 1998; Lasne *et al.*, 2002, 2007b). An epoetin produced in baby hamster kidney cells, epoetin omega, differed somewhat from epoetins alfa and beta in the glycosylation profile. However, this agent is no longer distributed. As patents for epoetins alfa and beta have expired, rHuEpo FOBs, have been approved by the European Medicines Agency (EMEA) including Binocrit (Sandoz International GmbH, Holzkirchen, Germany), Epoetin alfa HEXAL (Hexal Biotech Forschungs, Holzkirchen, Germany) and Abseamed (Medice Arzneimittel Puetter, Iserlohn, Germany). Epoetin delta (Dynepo; Shire, Hampshire, UK), which is produced from an engineered human fibrosarcoma cell line HT1080, has been described (Martin, 2007). These all can have minor differences on glycosylation profiles that must be considered in establishing positive test criteria, however, each can be differentiated from eEpo.

In contrast to these agents, rHuEpo is also manufactured and distributed in countries where less oversight on drug manufacturing results in significant product quality and structural differences from the existing commercial products Epoetins alfa and beta. Analysis of a number of rHuEpo preparations collected from third-world countries revealed characteristics that can vary considerably from each other and from marketed Epoetins alfa and beta. However, they can still be differentiated from eEpo with the current urine IEF test (Schellekens, 2005), although criteria for reporting a positive test result with some of these FOBs may need to be modified.

Additional ESAs are in various stages of clinical development and may appear in the athletic arena. These include polyethylene glycol-conjugated epoetin beta (Peg-epoetin beta) (Macdougall, 2005) and an Epo mimetic peptide, Hematide. Peg-epoetin beta (Mircera, F. Hoffmann-La Roche Ltd, Basel, Switzerland) was recently approved by regulators in the European Union. Peg-epoetin beta has the same mechanism of action as rHuEpo (Epoetin alfa, Epoetin beta and FOBs) and darbepoetin alfa. However, it is larger than both because of the chemical attachment of the 30-kDa Peg polymer. Peg increases the serum half-life of the ESA because of the increased hydrodynamic size of the molecule (Yamaoka *et al.*, 1994). Tests for Peg-epoetin beta should be readily available because, in addition to its increased size, Peg-epoetin beta contains Epoetin beta and should have a similar IEF profile.

Hematide is a non-naturally occurring Epo mimetic peptide that binds to the Epo receptor in a manner similar to rHuEpo, thereby activating it (Connolly *et al.*, 2000; Fan *et al.*, 2006). The peptide was pegylated to increase serum half-life. Because it has an amino-acid sequence distinct from rHuEpo it can be differentiated from eEpo. This should aid in development of specific tests to detect its abuse.

Other ESAs have been described including Epo fusion proteins (rHuEpo-IL3, Epo-albumin, rHuEpo-PAI1, rHuEpo-Fc and rHuEpo dimers) (Weich *et al.*, 1993; Kuai *et al.*, 2000; Dalle *et al.*, 2001; Elliott *et al.*, 2004). These agents have been tested in preclinical (animal) models and show varying degrees of erythropoiesis stimulation. As of the writing of this article, however, none are currently in clinical development.

Gene therapy

One aim of gene therapy research has been to deliver EPO genes to humans to treat their anaemia. Two strategies are in play including introduction of EPO genes into cells that are then implanted into the host, or direct transfer of genes to tissues such as muscle or bone marrow (Diamanti-Kandarakis *et al.*, 2005). The current methods of delivering genes to human cells use viral vectors, whereby the EPO gene expression is under control of a constitutive active promoter. Expression of the EPO gene is typically transient because the genes are either lost due to death of the Epo-expressing cells

or silenced (Lippin *et al*., 2005). Attempts to control expression of the gene have been attempted with some success; for example, through use of hypoxia responsive promoters (Binley *et al*., 2002), or by hybrid promoters responsive to small molecule activators (Rivera *et al*., 2005).

The fear that *EPO* gene therapy may be used for doping, while real, may not be of immediate concern due to safety and technical issues. The safety concerns are serious and include life-threatening erythrocytosis that can occur due to overexpression of the *EPO* gene (Zhou *et al*., 1998). There is also a report that Cynomolgus macaque monkeys with their anterior tibialis muscle cells engineered to express monkey Epo initially showed excessive erythrocytosis and then developed life-threatening pure red cell aplasia due to an immune response mounted against the monkey Epo protein (Chenuaud *et al*., 2004). The reason for the generation of an immunogenic product is unknown.

Detection of Epo for drug testing when made by gene therapy may be possible. Lasne *et al*. (2004b) reported that a monkey whose skeletal muscles were engineered to express monkey Epo had an IEF pattern that was more basic compared with the eEpo and more closely matched that of the rHuEpo made in Chinese hamster ovary or baby hamster kidney cells. This suggested that tests similar to the urine test used to detect doping with the Epoetins alfa and beta could be developed that could directly detect Epo made by gene therapy.

Conclusion

Abuse of medicines and procedures, and manipulation of the genome to enhance performance in competitive sport are widely frowned upon because of the damage to the athletes, the sport and the manufacturers who make them. However, the ergogenic benefits of increasing O_2-carrying capacity is well understood, particularly in patients who are anaemic. The existing tests and successful development of new tests will discourage misuse. Other strategies, such as more effective out-of-competition testing rules or longer term storage of blood or urine samples, with allowances for future testing, should also be considered. The latter strategy takes advantage of future improvements in testing procedures. However, additional steps will likely be required, including more effective education campaigns and policing by athletic organizations.

Conflict of interest

The author is an employee and stockholder of Amgen Inc., a biotechnology company that manufactures, distributes and markets erythropoiesis-stimulating agents.

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