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NOVEL ERYTHROPOIETIC AGENTS: A THREAT TO SPORTSMANSHIP

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Abstract

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The mass of hemoglobin (Hb) is an important determinant of aerobic power. Red blood cell production is stimulated by the glycoprotein erythropoietin (EPO). Recombinant human EPO (rHuEPO) engineered in Chinese hamster ovary (CHO) cell cultures (Epoetin alfa and Epoetin beta) and its hyperglycosylated analogue Darbepoetin alfa are known to be misused by athletes. The drugs can be detected in urine by isoelectric focusing, because the pattern of their glycans differs from that of endogenous EPO. However, doping control is becoming much more difficult, as various novel erythropoiesis stimulating agents (ESAs) are - or are to come - on the market. Gene-activated EPO (Epoetin delta) from human fibrosarcoma cells (HT-1080) has been approved in the European Union. rHuEPO (Epoetin omega) produced in transformed baby hamster kidney (BHK) cells is also available. ESAs to come include Biosimilars of the established Epoetins, long-acting pegylated Epoetin beta (CERA), EPO fusion proteins, synthetic erythropoiesis stimulating protein (SEP) and peptidic (Hematide) as well as non-peptidic EPO mimetics. Furthermore, small orally active drugs that are capable of stimulating endogenous EPO production are in preclinical or clinical trials. These include stabilizers of the hypoxia-inducible transcription factors (HIF) that bind to the EPO gene enhancer and GATA inhibitors which prevent GATA from suppressing the EPO gene promoter. It is crucial to inform the athletes and their supporting staff on the potential health risks.

Key words: *erythropoietin, erythropoiesis, recombinant DNA-technology, exercise, doping*

Introduction

In endurance sports – such as long-distance running, cycling and skiing – performance relies on an adequate O_2-supply to the heart and skeletal muscles. Hence, the rate of maximal O_2-uptake ($\dot{V}O_2$ max) is an important determinant of the aerobic power. $\dot{V}O_2$ max correlates with the O_2-carrying capacity of the blood. The O_2-capacity of the blood increases with the blood hemoglobin concentration (Hb). Thus, within certain limits, higher Hb levels are associated with improved performance. Apart from the O_2-content of the arterial blood the aerobic power increases with the maximal heart rate and stroke volume, and the myoglobin content and activities of mitochondrial enzymes of the muscles (Fig. 1).

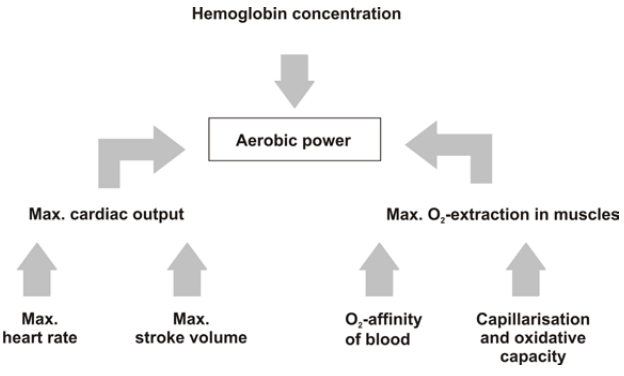

Fig. 1. *Parameters determining the aerobic power of a person (modified from (68))*

Red blood cell production is regulated by the glycoprotein hormone erythropoietin (EPO). EPO gene expression is controlled by feedback mechanisms involving the tissue O_2-pressure (pO_2), which depends on the Hb concentration, the arterial pO_2, the O_2-affinity of the blood and the rate of blood flow. After birth the kidney becomes the main EPO producing organ. EPO gene expression is under the control of several transcription factors (Fig. 2). The 5' promoter possesses GATA binding sites (1). GATA-2 has been reported to inhibit EPO gene expression (2-4). In addition, the EPO promoter is thought to be suppressed by NF-κB (5). The hypoxia-inducible EPO enhancer which is a 50-base pair element located 3' of the EPO gene contains at least two transcription factor binding sites. The proximal site of the EPO enhancer binds the hypoxia-inducible factors-1 or -2 (HIF-1 or -2) which

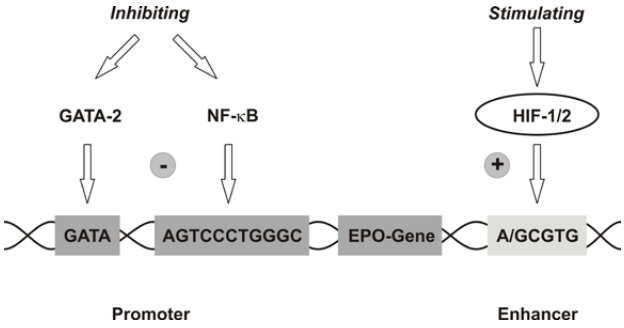

Fig. 2. *Transcriptional factors that suppress (-) the EPO promoter or activate (+) the EPO enhancer, respectively*

act as transcriptional activators (6). The distal site of the enhancer consists of a direct repeat of two nuclear hormone receptor half sites for hepatocyte nuclear factor-4α (HNF-4α) which cooperates with HIF (7).

Although GATA-2 binding to the EPO promoter is reduced on hypoxic stress (2, 3), the main mechanism by which hypoxia stimulates the expression of the EPO gene is binding of HIF. Recent studies indicate that HIF-2 (rather than HIF-1) is the primary transcription factor inducing EPO expression in hypoxia (8, 9). The HIFs are heterodimeric proteins composed of an O_2-labile α-subunit and a constitutive β-subunit (10). The C-terminus of the HIF-α subunits possesses O_2-dependent degradation domains (O-DDD), which contain O_2-sensitive prolyl and asparaginyl residues. Catalyzed by specific prolyl-4-hydroxylases (HIF-PHD) distinct prolyl residues (Pro^{402} and Pro^{564} in human HIF-1α, Pro^{405} and Pro^{531} in human HIF-2α) are hydroxylated in the presence of O_2, Fe^{2+} and 2-oxoglutarate (for references see Bruegge et al. (11)). Prolyl hydroxylated HIF-α combines with the von Hippel-Lindau tumor suppressor protein (pVHL) to form complexes that are polyubiquitinated by an E3-ligase and undergo immediate proteasomal degradation. The transcriptional activity of the HIFs is suppressed by another O_2-dependent hydroxylation, namely at Asn^{803} in human HIF-1α and Asn^{847} in human HIF-2α. This reaction is catalyzed by a HIF-α specific asparaginyl hydroxylase that is also termed „factor-inhibiting HIF-1" (FIH-1) (12, 13). On asparaginyl hydroxylation the binding of the co-activator CBP/p300 to the transactivating domain of HIF-α is prevented (12, 14, 15). Like the PHDs, FIH-1 is an Fe^{2+} containing and α-oxoglutarate requiring dioxygenase.

The HIF transcription factors do not only activate the EPO gene. More than 100 genes have been identified that are induced by binding of HIF to hypoxia-response elements (HRE), including the genes encoding the glucose transporters 1 and 3, several glycolytic enzymes and vascular endothelial growth factor (VEGF) (16, 17). Little is known about the consequences of the activation of these genes by HIFs in regard to physical performance.

Because the tissue pO_2 is the controlled variable, EPO gene expression will not only be stimulated when the O_2 capacity of the blood is lowered (anemia) but also on residence at high altitude (hypoxemia). On ascent to altitude serum EPO reaches peak values after about one day and then falls to a new plateau that is moderately above sea-level value (18, 19). The stimulation of EPO production by hypoxemia has been used by sportsmen to improve performance. Living and/or training at altitude may result in augmented erythropoiesis, a lowered O_2-affinity of red blood cells due to an increase in erythrocytic 2,3-disphosphoglycerate levels, and an improved oxidative capacity of muscles (20-23). Ethically questionable, high altitude residence can be mimicked at sea-level by living in tents or rooms with reduced O_2-concentrations (for references, see (24)). However, not all authors have confirmed that living under conditions of moderate hypoxia leads to increases in Hb and $\dot{V}O_2max$ (25).

Physiology of EPO

Tissue hypoxia is the main stimulus of EPO production in the kidneys and other organs (for references see (26)). In situ hybridization studies have identified a subgroup of peritubular fibroblasts as the site of EPO gene expression in the kidney (27). Human EPO is an acidic glycoprotein with a molecular mass of 30.4 kDa. Its peptide chain consists of 165 amino acids which form two bisulfide bridges. The carbohydrate portion (40% of the molecule) consists of 3 tetra-antennary N-linked (at Asn^{24}, Asn^{38} and Asn^{83}) and one small O-linked (at Ser^{126}) glycans (28). Like other plasma glycoproteins EPO circulates as a pool of isoforms that differ in glycosylation and biological activity (29-31). The N-glycans, which possess terminal sialic acid residues, are essential for the *in vivo* biological activity of EPO (32). De-sialylated EPO is rapidly removed from the circulation. *Vice versa,* the introduction of additional N-glycans into recombinant EPO by genetic engineering results in EPO analogues with prolonged *in vivo* survival (33).

The principal targets of EPO are erythrocytic progenitors in the bone marrow, particularly the colony-forming units-erythroid (CFU-Es). The erythrocytic human EPO receptor (EPO-R) is a 484 amino acid membrane protein. Its calculated mass of 52.6 kDa is increased to about 60 kDa due to glycosylation and phosphorylation (34). One EPO molecule binds to two EPO-R molecules which constitute a homodimer (35, 36). With a view to the novel erythropoiesis stimulating agents (ESA) it is noteworthy that the affinity of EPO analogues for EPO-R decreases with the degree of glycosylation of EPO (37). On EPO binding to its receptor intracellular signaling pathways are activated that prevent the programmed cell death of the erythrocytic progenitors and promote their proliferation and differentiation.

The normally low concentration of EPO enables only a small percentage of erythrocytic progenitors to survive and to proliferate (38). However, when the concentration of EPO rises in blood, either endogenously or following the administration of ESA, more CFU-Es escape from apoptosis and give rise to morphologically identifiable proerythroblasts and normoblasts. The developmental time from a CFU-E to its reticulocytic progeny is several days and involves 4-6 cell devisions. Thus, it takes about 3-4 days before significant reticulocytosis becomes apparent after an acute increase in plasma EPO. The action of EPO on

the myeloid erythrocytic progenitors is augmented by other hormones, f.e. by androgens (39). Androgenic steroids were earlier used clinically for treatment of severe anemia (40).

EPO therapy in renal failure

Lack of EPO is the primary reason for the development of anemia in chronic kidney disease (CKD). Recombinant human EPO (rHuEPO) was introduced as an antianemic drug for treatment of CKD patients 20 years ago (41, 42). The first-generation rHuEPO preparations (Epoetin alfa and Epoetin beta) have been engineered in cultures of transformed Chinese hamster ovary (CHO) cells that carry cDNA encoding human EPO (43). In addition, rHuEPO (Epoetin omega) engineered in baby hamster kidney (BHK) cell cultures has been used clinically in some Eastern European, Central American and Asian countries (44-46). In view of the relationship between the number and integrity of the N-glycans and the *in vivo* stability of EPO a CHO-cell derived hyperglycosylated rHuEPO analogue (Darbepoetin alfa) has been developed, which is increasingly administered to CKD patients. This compound possesses two extra N-linked carbohydrate chains as a result of site-directed mutagenesis for exchange of five amino acids. Compared to the Epoetins, which have a plasma half-life of 6-8 h, Darbepoetin alfa has a 3-fold longer half-life (37). The biological activity of 200 U (units) *per* µg rHuEPO peptide core corresponds to that of 1 µg Darbepoetin alfa peptide (47). In addition to the anemia of CKD, the anemias associated with cancer, myelodysplastic syndromes, bone marrow transplantation, autoimmune diseases, AIDS, hepatitis C and heart failure can be prevented by treatment with rHuEPO or Darbepoetin alfa (48). In addition, the drugs have been administered in the surgical setting to stimulate erythropoiesis in phlebotomy programs for autologous re-donation or for correction of a pre- or postoperative state of anemia. Note that it is recommended that the doses of ESAs should be adjusted in general for each patient (including renal failure patients, cancer patients receiving chemotherapy and patients receiving ESAs pre-operatively for reduction of allogeneic red blood cell transfusion) to achieve and maintain the lowest Hb level sufficient to avoid the need for red blood cell transfusion and not exceed 120 g/l. At higher Hb levels, ESA therapy may be associated with an increased risk for serious cardiovascular events (thromboembolism) and death.

Blood doping

According to the World Anti-Doping Agency (WADA) blood doping is the misuse of certain techniques and/or substances to increase one's red blood cell mass, which allows the body to transport more O_2 to muscles and therefore increase stamina and performance. Indeed, it has been known for decades that induced erythrocythemia will increase aerobic work capacity. F.e., almost 30 years ago Buick et al. (49) showed in a double-blind design that autologous re-infusion of approximately 900 ml of freeze-preserved blood increases $\dot{V}O_2max$ and running time to exhaustion in highly trained runners. Likewise, it has been shown that re-infusion of autologous blood stored in a refrigerator for 4 weeks after phlebotomy significantly increases performance in cross-country skiing (50). Clearly, the transfusion of blood to improve endurance performance of athletes during training or competition is forbidden (51). While homologous blood doping (transfusion of compatible blood taken from another person) is detectable by flow cytometry ((52); the test was implemented at the 2004 Summer Olympic Games in Athens), autologous blood doping (transfusion of one's own blood) cannot be detected at present by direct measures. However, the donor of red cells stored for autologous re-transfusion can be identified by comparative blood group-typing and other immunological markers. The apparent recent resurgence of blood transfusion for doping is a likely consequence of the introduction of the method for demonstration of rHuEPO in urinary samples in 2000 (53). Attempts may also be made to increase the O_2-capacity of the blood by use of hemoglobin-based O_2 carriers (HBOCs). However, HBOCs can be detected by electrophoretic methods (54) or size-exclusion HPLC (55).

Immediately after rHuEPO became available as an erythropoiesis-stimulating drug it has been imputed to be abused by athletes in aerobic sports (56, 57). There is suspicion that rHuEPO-induced erythrocytosis caused the deaths of 18 world-class Dutch and Belgian cyclists (57), although it has remained unproven that they were truly treated with rHuEPO. Blood viscosity and cardiac afterload increase with increasing hematocrit (Hct), while cerebral blood flow decreases (58). The main risks of erythrocytosis include heart failure, myocardial infarction, sizes, peripheral thromboembolic events and pulmonary embolism. The risks are raised during the competition when the blood viscosity increases further due to intensified perspiration and the shift of fluid from the intravascular into the interstitial space (for references, see (59)). The fact that the above mentioned cases of death of cyclists suspected of rHuEPO doping did not occur during exercise but during periods of physical inactivity does not militate against the detrimental effect of erythrocytosis, because blood flow in the microcirculation will slow down during physical inactivity thereby favoring the development of thrombi. The risk to promote tumor growth by EPO doping has been considered (60), although clinical evidence supporting this fear is missing.

A study in healthy male students in physical education who were moderately to well trained has shown that a low dose of rHuEPO (about 30 U/kg) injected subcutaneously three times a week increases $\dot{V}O_2$ max and physical performance, measured as time to exhaustion on a standard treadmill running test, along with the increase in Hb and Hct (61). These findings have been confirmed in other studies (62-65). rHuEPO treatment increases red cell mass in healthy humans, while blood plasma volume decreases, with both effects contributing to the elevation in Hb (66). For the most part, rHuEPO doping will be more effective than hypoxia exposure (67). In addition, a psychological investigation has shown that the perception of increased physical condition can lead to a stronger commitment to training. The authors have pointed out that the administration of rHuEPO is associated with a potentially dangerous hedonic effect linked to endurance training (65).

It should be noted that physical exercise *per se* does not have a major influence on circulating EPO levels as has been studied in various disciplines (for references see (68)). Despite the lack of an increase in EPO production in response to acute physical work, the number of reticulocytes may increase within 1 to 2 days after exercise (69). This reaction is likely caused by stress hormones such as catecholamines and cortisol which stimulate the release of young red blood cells from bone marrow. Note that Hb levels and Hct are often below normal in athletes (59). This so-called „sports anemia" is a pseudoanemia as it results from an increase in the blood plasma volume.

The exclusion from competition of athletes with abnormally high Hb or Hct values by some sport organizations (f.e.: UCI, FIS, ISU, IBU) is justified on medical prophylactic grounds. However, it is by no means proof of rHuEPO doping, because Hb levels in unmanipulated persons often exceed the limits set by sport organizations (see Fig. 3). In addition, Fig. 3 shows that there is a large variation in the normal levels of Hb and serum EPO in healthy humans.

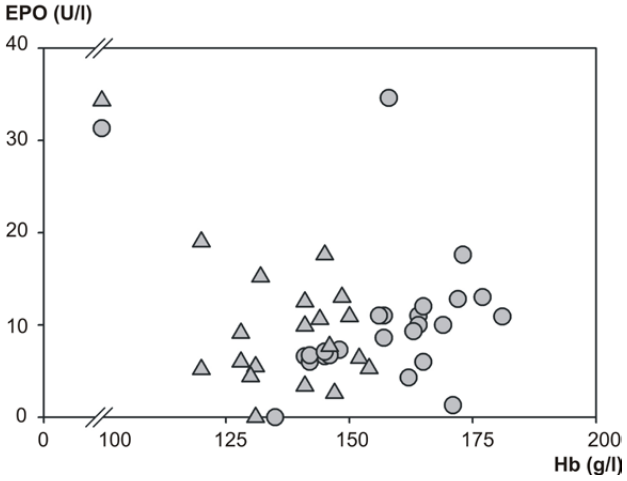

Fig. 3. *Serum EPO related to blood Hb concentrations in healthy humans (n = 43, laboratory staff (125))*

Detection of Epoetin and Darbepoetin doping

An indirect screening method for inspection of the misuse of Epoetin or Darbepoetin by athletes has been established which is based on the values of Hct, reticulocytes, macrocytes and the concentrations of circulating EPO and soluble tranferrin receptor (70-72). If the result provides grounds for suspicion, misuse can be proven by direct demonstration of the drugs in urine samples. The use of charge differences between recombinant and endogenous EPO in doping control was first proposed by Wide et al. (73). In the current procedure, about 20 ml urine is approximately 1000-fold concentrated and depleted of small molecules by ultrafiltration. The samples are subjected to isoelectric focusing (pH 2 - 6) followed by immunodetection (53). Non-specific interaction with other urinary proteins is minimized by an additional Western blotting step, whereby the primary monoclonal anti-EPO antibody (AE7A5) is transferred to a new membrane, which is then incubated with labeled anti-mouse immunoglobulin secondary antibody (74). Technical details of the procedure are described in a WADA document (75).

The EPO isoforms pattern of urine from untreated control subjects exhibits about 10 bands in the range pI 3.77 – 4.70 on immunoblots. Blots from subjects treated with Epoetin alfa, Epoetin beta or Epoetin omega contain more basic bands. Interestingly, when 14 frozen samples of urine with relatively high concentrations of immunoreactive EPO from participants in the Tour de France 1998 were subjected to isoelectric focusing after the method was newly developed, all of the samples exhibited a banding pattern typical of rHuEPO (53). Darbepoetin alfa migrates more in the acidic range than endogenous EPO (76, 77). The computer program GASepo (78), which is freely available for anti-doping laboratories, enables it to standardize the results by means of image segmentation. Methodological weaknesses of the detection of recombinant ESA have been described elsewhere (79-81). To overcome some of the problems (such as time-consuming urine sample preparation, low sample load capacity and non-specific binding) an improved concentration technique in combination with 2D electrophoresis has been developed (82). A novel problem is suspected adding of proteases by athletes to their urinary samples, which destroys the erythropoietic proteins. The detection of exogenous proteases is possible by mass spectrometry.

It has been suggested to establish individual „Hematologic passports" for elite athletes which would enable one to detect sudden unphysiological changes in hematologic parameters. However, whether such passports, which also include data on the total Hb mass as determined by CO re-breathing (83), is useful in practice still needs to be clarified. The significance

of such data in law is questionable. In addition, this procedure does not appear appropriate in leisure sports and in elite sport disciplines, in which the competitors have a short history until success.

Novel Epoetins

The WHO Expert Committee for International Nonproprietary Names (INN) defines „Epoetins" as products that are characterized by an amino acid sequence similar to that of endogenous EPO (84). Greek letters are added to differentiate between compounds varying in the glycosylation pattern.

Epoetin delta is a novel recombinant EPO for treatment of CKD patients (85, 86) that was approved by the European Medicines Agency (EMEA) in 2002 and first marketed, in Germany, in 2007. Epoetin delta is engineered in human fibrosarcoma cell cultures (line HT-1080). The product is also called gene activated EPO (GA-EPO) because the expression of the native human EPO gene is activated by transformation of the cells with the cytomegalovirus (CMV) promoter (87). In contrast to CHO or BHK cell-derived rHuEPO, Epoetin delta does not possess N-glycolylneuraminic acid (Neu5Gc) because – in contrast to other mammals including great apes – humans are genetically unable to produce Neu5Gc due to an evolutionary mutation (88). Nevertheless, it is unlikely that the behavior of Epoetin delta on isoelectric focusing is identical to that of native human urinary EPO because the structure of N-glycans is not only species- but also tissue-specific. In addition, the pattern of isoforms is influenced by the environmental respectively cell culture conditions and the purification procedures applied. In fact, earlier studies have shown that rHuEPO from a human lymphoblastoid cell line (RPMI 1788) transfected with the human EPO gene possesses N-glycans with unusual characteristics (89). Support for the expectation that Epoetin delta doping may be detectable comes also from observations following homologous EPO gene transfer. *In vivo* EPO gene transfer in skeletal muscle in macaques results in EPO which differs in isoelectric pattern from the endogenous EPO of the animals as demonstrated by isoelectric focusing (90).

Since the patents of Epoetin alfa and Epoetin beta have expired recently in several countries and because the market for rHuEPO is very lucrative, copies of the established rHuEPO preparations are expected to be marketed soon, respectively are already on the market. These products are named „Biosimilars" in the EU and „Follow-on Biologics" in the USA. Outside the EU and the USA copy rHuEPOs are already produced by companies other than the innovators and clinically used as anti-anemic drugs. F.e., the therapeutic efficacy of a CHO cell-derived rHuEPO produced in Havana, Cuba, has been proven (91). Apart from this single report, however, little information is available on the structure, pharmacodynamics and pharmacokinetics of copy rHuEPO preparations. Because the glycosylation pattern of rHuEPO depends on the cell line, the culture conditions and the product purification procedures it is likely that all copy rHuEPOs differ with respect to the structure of their N-glycans. Indeed, when 11 samples of Epoetin products marketed outside of Europe and the USA were obtained from 8 different manufacturers were investigated for their behavior on isoelectric focusing major differences between the products of different manufacturers as well as between batches of products of the same manufacturer were detected (92). Clearly, the availability of rHuEPO preparations with isoforms that are more acidic as well as such with isoforms that are more basic than endogenous EPO will pose major problems with respect to doping analysis, in particular if the products are used at low dosing and in combination. In addition, it is reasonable to imagine irresponsible athletes and trainers who have made use of counterfeit Epoetin preparations, which have cropped up in the USA (93) and elsewhere. Subjects prone to misuse copy or counterfeit Epoetins should be aware that such products do not always meet the self-declared specifications and the common purity standards (94).

Another new Epoetin derivate in clinical trials is CERA (Continuous Erythropoiesis Receptor Activator, Ro 50-3821). The molecular mass (60 kDa) of CERA is about twice that of the Epoetins, because a methoxy-polyethylene glycol polymer (PEG) is integrated *via* amide bonds between the N-terminal amino group of alanine (Ala^1) and the ε-amino groups of lysine (Lys^{45} or Lys^{52}) by means of a succinimidyl butanoic acid linker (95). As it is known from pharmacokinetic studies of other pegylated therapeutic proteins (96, 97), the half-life of circulating CERA is prolonged (to about 6 days) compared to that of the conventional Epoetins alfa and beta (98). Details of the sites and mechanisms of the clearance of CERA still need to be described. Apart from CERA, pegylated Epoetin alfa (99) and a pegylated rHuEPO analogue (100) have been tested for their efficacy in experimental animals.

Several other EPO-like molecules and derivatives are in preclinical or clinical trials (Table 1). (i) A hyperglycosylated analogue of Darbepoetin alfa (AMG 114) with a prolonged half-life is tested clinically. (ii) An interesting novel product is SEP (Synthetic Erythropoiesis Protein, 50 kDa) which is chemically synthesized as 166 amino acid protein (including the Arg^{166} that is cleaved before human EPO is secreted from cells) with covalently bound polymer moieties (101). The half-life of circulating SEP is probably longer than that of the Epoetins. The erythropoietic effect of SEPs has been shown to vary in experimental animals depending on the number and type of the at-

tached polymers (102). (iii) Recombinant EPO fusion proteins have been expressed which contain additional peptides at the carboxy-terminus to increase *in vivo* survival (103). (iiii) Large EPO fusion proteins (76 kDa) have been designed which are derived from cDNA encoding two human EPO molecules linked by small flexible polypeptides (104, 105). (iiiii) Still another approach is the genetic fusion of EPO with the Fc region of human IgG (106). Interestingly, an Fc-EPO fusion protein has been successfully administered in a phase I trial to human volunteers as an aerosol (107). With a view to the possible routes of administration, it should be noted that rHuEPO has been applicated by ultrasound-mediated transdermal uptake to humans (108) and by oral route in liposomes to rats (109).

Table 1. *Novel drugs for stimulating erythropoiesis*

Protein-based ESAs
Epoetin alfa, Epoetin beta, Epoetin omega
Darbepoetin alfa
Epoetin delta
Copy rHuEPOs, biosimilars and counterfeit products
CERA
SEP
EPO fusion proteins
Small molecule EPO mimetics
Peptides
Non-peptides
HIF stabilisers
Cobalt
α-Ketoglutarate competitors

EPO mimetics

About 10 years ago several small bisulfide-linked cyclic peptides composed of about 20 amino acids were identified by random phage display technology which are unrelated in sequence to EPO but bind to the EPO receptor and are erythropoietically active (35, 36). Subsequent studies showed that the potency of the EPO mimetic peptides (EMP) could be greatly increased by covalent peptide dimerization with a PEG linker (110). A potent EMP has been chosen to develop Hematide, a pegylated synthetic dimeric peptidic ESA, which stimulates erythropoiesis in experimental animals (111). The half-life of Hematide in monkeys ranges from 14 h to 60 h depending on the applicated dose (111). A phase I study in healthy male volunteers has shown that single injections of Hematide cause dose-dependent increases in the concentration of reticulocytes and Hb (112). Hematide is currently in phase II of its clinical trial program. Although Hematide can be detected by appropriate ELISA no data are available demonstrating Hematide in human urine.

Several non-peptide molecules capable of mimicking the effects of EPO have also been identified (113, 114) but the potential of these compounds to stimulate erythropoiesis in humans still needs to be proven.

HIF activators

Under normoxic conditions EPO gene expression is suppressed physiologically because the hypoxia-inducible transcription factors (HIF) are inactivated due to HIF-α prolyl and asparaginyl hydroxylation. The HIF-α hydroxylases do not only require O_2 for their catalytic action but also Fe^{2+} and 2-oxoglutarate (for references, see (11)). Accordingly, HIF-α hydroxylation can be prevented by iron depletion or by the application of 2-oxoglutarate analogues (Fig. 4).

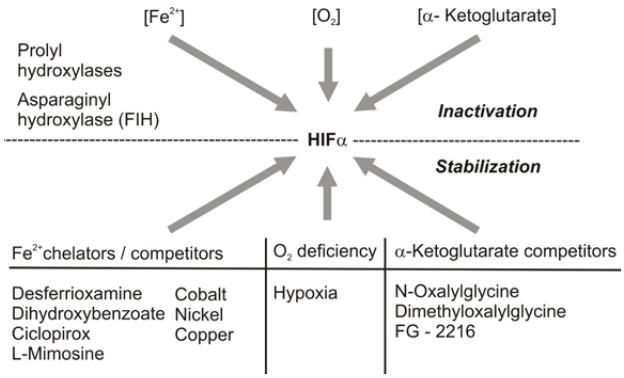

Fig. 4. *Compounds inducing endogenous EPO expression by inhibiting HIF-α hydroxylation in normoxia*

stabilize HIF-α (115-118) and promote EPO expression in cell cultures (117-119) as well as in humans *in vivo* (119, 120). However, iron chelators are not suited for stimulation of red cell production in humans in the long term because iron is required for heme synthesis. HIF-dependent EPO gene expression in normoxia can also be induced by divalent transition metals such as cobalt or nickel which – at leased partially – exert their action by displacing Fe^{2+} from the HIF-α hydroxylases (121, 122). In addition, cobalt appears to bind to HIF-2α directly, thereby preventing the normoxic degradation of HIF-2α (123). The stimulation of EPO production and erythropoiesis by cobaltous ions has been known for many years (124). Actually, the international EPO standard was originally calibrated against cobalt, with 1 unit (U) of EPO producing the same erythropoiesis-stimulating response in experimental animals as 5 μmol cobaltous chloride (for references see (125)). Prior to the availability of rHuEPO cobalt was used to treat anemic patients (126). Note that the use of cobalt as an anti-anemic drug is today obsolete because of its toxicity. Nevertheless, it has been speculated that

some athletes may misuse cobalt to stimulate EPO production (127).

With a view to doping, the 2-oxoglutarate analogues which stabilize HIF-α in normoxia („HIF-stabilizers") are a major problem. Many of these compounds were earlier tested *in vitro* (128) and *in vivo* (129, 130) for their inhibitory action on collagen prolyl 4-hydroxylases which also need 2-oxoglutarate as cofactor. The primary purpose of the initial studies was to develop drugs for treatment of fibrotic diseases (131, 132). Among the HIF-stabilizers are simple molecules like N-oxalylglycine, ethyl-3,4-dihydroxybenzoate or L-mimosine which are easy to synthetize and can be taken orally. 2-Oxoglutarate analogues clearly stimulate erythropoiesis *in vivo* (133). HIF-stabilizers have already been administered to healthy control subjects (134) and to patients with CKD (135) in clinical trials investigating novel strategies for the treatment of anemia. It should be noted, however, that the application of 2-oxoglutarate analogues to induce EPO expression will result in the expression of a great number of other genes which may result in serious unwanted effects. A major concern is the promotion of tumor growth.

Conclusion

In their worth reading reappraisal of the abuse of drugs by athletes Duntas and Parisis (136) have cited the ancient Greek saying „None so wretched as the competitor who wins victory through cheating". Yet the authors have also conceded that the commercialization has progressively changed the spirit of sport, as the desire to win at any cost has overcome all ethical and medical considerations. Success in elite sports gives promise of fame and financial rewards. Moreover, trainers and sport officials press their protégées for victories, and we (the spectators) long for heroes. There is little doubt that the novel erythropoiesis stimulating drugs will be picked up in attempts to improve performance. Unfortunately, misuse of drugs in sports is not restricted to elite sports but has also gained entry to leisure sports. Here, an additional health problem relates to the fact that amateur athletes will often act without medical control.

With several novel erythropoietic drugs being on the market doping control has become very difficult, not to say hopeless. The new drugs include various kinds of Epoetins, biosimilars, mutated EPO analogues, long-acting pegylated Epoetins and EPO analogues, EPO fusion proteins and peptidic as well as non-peptidic EPO mimetics. Compounds are at hand which act as stabilizers of hypoxia-inducible transcription factors (HIF) that bind to the EPO enhancer. GATA inhibitors are under development, which can be orally taken and prevent GATA from suppressing the EPO promoter (137;138). Because EPO signaling involves protein tyrosine phosphorylation, inhibitors of hemopoietic cell phosphatase (HCP) may also become available for misuse (139). The novel drugs are being developed primarily for therapeutic benefits, i.e. the alleviation of anemia in patients suffering from chronic renal failure or inflammatory or malignant diseases (140). Because there are not only major technical problems in detecting the drugs for proof of doping but also difficulties with respect to intention, moral and law, it is crucial to inform the athletes and their supporting staff of potential health risks, although this may be an act of „tilting at windmills".

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A – Study Design
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F – Literature Search
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