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Erythropoiesis-stimulating agents: development, detection and dangers

Stefan E. Franz*

Epoetin alfa, the first member of the family of erythropoiesis stimulating agents (ESAs), was introduced to the market in 1989. Since then development has progressed to epoetins of the third generation. Currently drugs that use alternative approaches to stimulate erythropoiesis are under development. Uptake of all available ESAs into doping has occurred rapidly after their introduction. A multitude of dangers to health are associated with the illicit use of these substances. Different approaches to detect ESAs in doping control have been developed to comply with the very diverse nature of the compounds used. Future developments in the field of ESA require the development of new techniques in doping analysis. This review gives an overview of the development of ESA and its detection methods as well as future developments. [Correction made here after initial online publication]

Keywords: doping; erythropoietin; erythropoiesis stimulating agents; doping detection

Introduction

Since the beginning of competitive athletics athletes have been searching for methods to improve performance. For endurance athletes the capacity to transport oxygen in the blood is a limiting factor when all other aspects of the cardiovascular system have been optimized. To overcome this limit, athletes started to experiment with blood transfusions in the early 1970s. Blood transfusions, however, require a complicated infrastructure and the number of possible infusions is limited. A quantum leap in the history of doping occurred, therefore, when recombinant erythropoietin and other erythropoiesis stimulating agents (ESAs), which boost production of erythrocytes, became available on the market.

Erythropoietin

Endogenous erythropoietin (Epo) is synthesized mainly in peritubular fibroblasts in the kidney.[1] Expression is controlled by constitutively expressed transcription factors (hypoxia induced factors – HIF). Under normoxic conditions HIFα is degraded through the influence of HIFα prolyl hydroxylases. Under hypoxic conditions HIFα remains stable and forms dimers with HIFβ. The HIF$\alpha\beta$ dimer leads to the transcription of Epo and a great number of other genes.[2] Erythropoietin prevents apoptosis of erythroid burst forming units and stimulates their differentiation into erythroid progenitor cells and finally erythrocytes.[3] Physiologically, increased oxygen delivery caused by an increase in erythrocytes will result in a negative feedback mechanism on the expression of erythropoietin. The human Epo molecule is an acidic glycoprotein that consists of a protein backbone of 165 amino acids, three N-linked and one O-linked glycan.[4] The molecular weight of the whole molecule is about 30 kDa depending on the amount of glycosylation. The glycans amount to about 40% of the whole weight. The N-linked glycans have a strong influence on pharmacokinetic and pharmacodynamic properties of the Epo molecule, namely the affinity to the receptor and serum half life.[5] The pattern of glycosylation is dependent on the species as well on the type of cell in the body. Serum Epo usually consists of several different isoforms, which can be separated by isoelectric focusing (IEF).[6]

Recombinant Erythropoietins and Analogues

Recombinant erythropoietins are produced by transfection of the erythropoietin gene into mammalian cells in culture or activation of the gene in human cells.[7,8] The amino acid sequence is similar in all recombinant erythropoietins (except for darbepoetin alfa) but the glycosylation patterns vary widely depending on the cell type, environmental parameters during culture and the purification process. Moreover, during the purification process only parts of the isoforms are isolated whereas others are discarded to ensure stable pharmacokinetics and pharmacodynamics of the final product.[9] These variations yield an IEF pattern that is different from the endogenous Epo and can therefore be used in doping analysis.

Epoetins were licensed by international regulatory bodies for therapeutic indications that include anaemia from chronic kidney disease, anaemia associated with tumours and chemotherapy treatment in oncological patients and elevation of hematocrit levels in preparation for autologous blood donation.[10] There is much experimental data showing epoetins to be beneficial in the treatment of diseases involving ischaemic or toxic cell death; the anti-apoptotic properties result in lower rates of cell death and smaller scars in affected organs.[11] These therapies remain experimental.

The first recombinant erythropoietin that was commercially available was epoetin alfa, which was marketed in 1989 by Amgen.[12] Like many other therapeutic proteins it was expressed

* *Correspondence to: Stefan E. Franz, Roche Pharma (Schweiz) AG, Schönmattstrasse 2, CH 4153 Reinach, Switzerland. E-mail: s.franz@gmx.ch*

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Roche Pharma (Schweiz) AG, Reinach, Switzerland

in Chinese hamster ovary (CHO) cells. Its serum half life is approximately 8 h when administered intravenously and about 24 h when given subcutaneously. In therapeutic use the frequency of administration ranges between one and three times per week.[13] Application via the subcutaneous route results in a lower dose requirement of about 30% compared with the intravenous route.[14] In 1990 Boehringer Mannheim (acquired by Roche in 1997) launched epoetin beta. Like epoetin alfa it was also expressed in CHO cells and even though glycosylation and biological activity of the molecule is different from epoetin alfa the molecule does not provide significantly different clinical characteristics.[15] Therefore epoetin alfa and beta together were termed 'first-generation' epoetins.

Since the late 1990s a multitude of producers in the emerging world have started to produce copies of the first-generation epoetins. At this time around 80 different kinds of epoetin analogues are being produced in Eastern Europe, Asia, Africa and the Americas.[16] Currently none of these are licensed by the Food and Drug Administration in the US or by the European Medicines Agency. These epoetins not only very often show a great variation in biological activity in comparison to other products but also wide intra-batch variability due to the absence of strict standardization of cultivation and purification procedures.[17,18] In some products contamination with bacterial endotoxins or other contaminants has been described.[19]

Since the patent for epoetin alfa expired in Europe in 2004, several companies specializing in generic pharmaceuticals have started to develop and market biosimilar epoetins (epoetin alfa, omega and zeta).[20] Studies were performed to compare the pharmacokinetics and efficacy of the biosimilars with original epoetins.[21,22,23] Available clinical data suggest that biosimilar epoetins show clinical properties that are comparable to those of the original molecules, even though variations in the manufacturing process as compared to the original manufacturers result in differences of glycolysation. The original epoetin alfa was not available as a comparator drug for subcutaneous application during the development phase of the biosimilars due to the occurrence of antibody-mediated pure red-cell aplasia (PRCA) – a progressive decline in the number of circulating red blood cells due to missing production or destruction of precursor cells)[24] – so there are no data on the safety of administration by this route and the drugs are not licensed for subcutaneous injection in patients with chronic renal failure.[25,26]

In 2007 Shire introduced epoetin delta to the market. This epoetin showed pharmacokinetic properties very similar to epoetin alfa and therefore similar clinical properties.[8,27,28] Nevertheless it was clearly distinct from other epoetins in that it was produced by transfection of a Cytomegalovirus (CMV) promoter into a human fibrosarcoma cell. The choice of a human cell line resulted in a glycosylation pattern that was more similar to endogenous erythropoietin and clearly different from epoetins expressed in hamster cells.[29] In 2008 epoetin delta was taken off the market for economic reasons.[30]

To overcome the pharmacokinetic limitations of the first-generation of epoetins, research was targeted to develop molecules with a longer serum half life and to achieve longer application intervals. The first candidate to achieve these goals was darbepoetin alfa. Through site-directed mutagenesis of five amino acid residues in the protein backbone of erythropoietin it was possible to introduce two additional N-linked glycans.[31] The molecular weight of darbepotin alfa is approximately 38 kDa. The two side chains resulted in an increase of the serum half life to about 24 and 48 h when administered intravenously or subcutaneously, respectively.[32] This increase in half life results in a prolongation of administration intervals to once weekly or once every other week. In selected patient populations, dosing intervals of up to once monthly have been described.[33,34]

A next step towards more physiological pharmacokinetics was the development of methoxy polyethylene glycol-epoetin beta named continuous erythropoietin receptor activator (C.E.R.A.).[35] The principle of prolonging biological half life of therapeutic proteins by binding of a polyethylene glycol (PEG) molecule is a well established technique (i.e. PEG–Interferon alfa).[36] Several binding sites and several sizes of the PEG molecule were tested for optimal results regarding pharmacokinetics of C.E.R.A. The final result of this development was a molecule where a 30 kDa PEG molecule was covalently bound to epoetin beta. The size of the complete molecule, 60 kDa, is thus roughly doubled in comparison with endogenous erythropoietin and first-generation epoetins. The addition of a PEG molecule to epoetin beta leads to a decrease in affinity to the Epo receptor and a faster dissociation from the receptor, probably leading to less internalization into cells and a longer serum half life. The serum half life of C.E.R.A. has been shown to be about 135 h, irrespective of route of administration. The increased size of the molecule exceeds that of proteins readily filtered by the kidney. In clinical practice the pharmacokinetics of C.E.R.A. allow the routine administration of the drug, once monthly or even less frequently.[16]

Other forms of PEGylated epoetins and epoetins bound to albumin have been reported to be under development. Given the very competitive market, it is unclear whether these agents will become commercially available.

Other Erythropoiesis-Stimulating Agents (Esas): Future Directions

Two classes of nonepoetin ESA are most likely to become commercially available in the near future: Epo mimetic peptides and HIF-stabilizers.

In 1996 Wrighton *et al*. described a peptide identified by a peptide phage library that binds and activates the Epo receptor without showing homology with Epo.[37] This 20 amino-acid peptide showed only low *in vivo* activity, probably due to fast clearance from the organism. A construct of two peptides bound to two ends of a PEG molecule showed vastly enhanced biological activity compared with the monomer.[38] Affymax further explored this approach and designed another peptide that was linked to PEG, which was named Hematide.[39] Hematide shows a serum half life of about 20 h in healthy volunteers and peak reticulocyte counts around day seven.[40] Elevated haemoglobin levels were preserved for about one month. One important characteristic of Hematide is the absence of crossreactivity with antibodies against Epo.[41] This could possibly be therapeutic in cases of PRCA. The formation of anti Hematide antibodies would not lead to PRCA because remaining endogenous Epo levels would not be compromised.

HIF-stabilizers act through the inhibition of the prolyl hydroxylation of HIFα leading to ubiquitination and degradation in the proteasome. Fibrogen has developed a compound termed FG-2216 that inhibits HIFα hydroxylation and leads to an increase of reticulocytes and an increase of haemoglobin. Interestingly the elevation in serum Epo levels during treatment with FG-2216 is lower than expected hinting to the involvement of other genes regulated by HIF.[42] A very distinctive feature of HIF stabilizers is application via the oral route.

Clinical Dangers of Doping with Drugs Enhancing Haemoglobin Levels

Under physiological conditions haemoglobin levels are tightly controlled by blood oxygen content via the HIF system. An increase in haemoglobin levels increases the capacity of the blood to transport oxygen but the concurrent increase in erythrocytes increases the viscosity of the blood, compromising microcirculation and increasing the probability of thrombembolic events. While many professional athletes are tightly controlled to prevent hematocrit levels above 50%, which would lead to disqualification from competition, amateur athletes might use such compounds without laboratory controls, placing them in danger of reaching critical hematocrit levels. Even professional athletes monitored for their hematocrit levels might reach critical hematocrit levels during competition in hot weather if there is insufficient fluid replacement. Furthermore the bradycardia present in elite athletes might be attenuated during sleep and low flow velocities might lead to thrombembolic events.[43] In patients treated for renal anaemia it has been shown that, in the presence of functional iron deficiency, treatment with epoetin can result in elevation of thrombocyte counts and subsequent higher risk of cardiovascular events.[44] On the other hand, treatment with iron to enhance the effect of ESA might lead to iron overload.

Another concern is the origin of the drugs used by athletes. Not all of them have the infrastructure to get access to drugs from well controlled sources so they might get drugs of questionable provenance over the Internet. As mentioned above, some of the 'copy' epoetins manufactured in the emerging world are produced with insufficient quality control and might contain contaminants and show high intrabatch variability in biological activity, leading to poorly controllable hematocrit levels. Contamination and other variabilities might lead to an increase in immunogenicity that could cause PRCA. These dangers might be aggravated by improper cooling during shipping, especially with epoetin. The fact that even professional athletes obtain their drugs from dubious sources has been proven by confession of athletes. Biosimilar epoetins are currently not registered for subcutaneous use for safety reasons. It is unlikely that this limitation will be respected by illicit users.

Even though Epo mimetic peptides and HIF stabilizers have not gained entrance to the market, it is likely that these substances might be used shortly after or even before introduction to the market. Since long-term experience with drugs during development is only gained in specific subpopulations, use of these drugs in healthy individuals might show unexpected side effects. In HIF stabilizers concerns about long-term use have been raised because more than 100 genes are known to be regulated by HIF. Some of these genes play a role in carcinogenesis and neovascularization. Unfortunately it is very likely that the use of limited doping tests might make these substances very attractive for athletes who have been shown to be 'early adopters' in the past, despite possibly grave consequences. First approaches, however, were reported on the mass spectrometric characterization of model HIF stabilizers, which can be applied for doping-control purposes.[45]

Testing Procedures and Limitations

In doping analysis, the proteins contained in the athletes' urine are commonly concentrated by ultracentrifugation. The resulting retentate is separated on an IEF gel with a pH gradient usually ranging from 2 to 6.[46] During IEF, different isoforms form bands

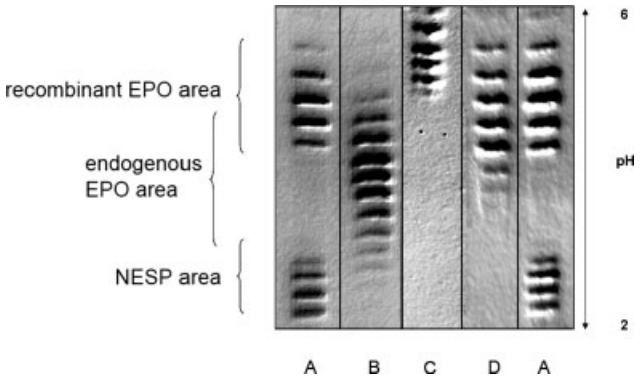

Figure 1. Differences in isoelectric focusing of different forms of epoetins and analogues in urine. Lane A: reference marker, which is a mix of epoetin beta and alfa (upper bands) and darbepoetin alfa (lower bands). Lane B: endogenous Epo. Lane C: C.E.R.A. Lane D: epoetin delta. [Correction made here after initial online publication]
Source: Figure courtesy of Prof. W. Schänzer, German Sports University, Cologne, Germany.

at the pH corresponding to their isoelectric point. Isoforms are then double-blotted with an erythropoietin antibody and visualized by a chemoluminescent process. Since the protein component of endogenous and the different recombinant forms of erythropoietins and analogues (with the exception of darbepoetin) is identical, the differences in the IEF patterns depend on glycolysation or addition of special side chains. The main isoforms of commercially available forms of erythropoietin (alfa, beta, zeta and omega) are focused in a more basic region than endogenous Epo (Figure 1) and can be identified thereby. Darbepoetin with its two additional side chains is focused in a more acidic range of the gel also clearly distinct from endogenous Epo. Since epoetin delta is generated in a human cell line it was proposed that glycolysation might be similar to endogenous Epo and provide an identical pattern in IEF. Actually epoetin delta provides a IEF pattern that is different from endogenous Epo but also shows some more acidic bands, which partially overlap with the endogenous window.[29,47] Inconclusive results in routine testing based on IEF are therefore possible. It has been shown that endogenous Epo shows a slightly lower molecular weight than recombinant epoetins in SDS.[48,49] In 2008 Kohler *et al*. published a new method to identify Dynepo in urine samples.[50] The basis of this assay is the lower relative mobility of Dynepo compared to endogenous erythropoietin when compared to Aranesp and recombinant rat Epo on a SDS-Gel. Rat Epo and Aranesp are run on an SDS gel together with the analytical sample and the ratio between distances from Aranesp to the analyte to distance of Aranesp to rat Epo is determined. In this assay the relative mobility of epoetin delta falls within the range of other recombinant epoetins and can be clearly distinguished from endogenous erythropoietin.[45]

Continuous erythropoietin receptor activator plays a special role in doping analysis. In theory, due to the covalently bound PEG molecule, it runs in an even more basic region on IEF in comparison to recombinant epoetins. Initial problems in doping analysis resulted from the fact, that routine test for epoetins were performed in urine. Due to its high molecular weight (~60 kDa) C.E.R.A. is not readily filtered in the kidney.[51] Only in physiological states that are accompanied by an altered state of glomerular filtration (proteinuria) can C.E.R.A. be detected in urine. This is the case in exercise-induced proteinuria occurring after strenuous exercise.[52] This makes testing feasible after competition but

might be negative in between competition phases where lower intensity of training occurs, so Lasne *et al*. modified the technique of IEF and double-blotting to be used in serum.[46] With this technique C.E.R.A. is clearly detectable. It is also possible to detect C.E.R.A. by the use of an antibody directed against the PEG portion of the molecule. An ELISA assay that the manufacturer used in pharmacokinetic studies during development was provided to the World Anti-Doping Agency (WADA). In this assay Epo is immobilized by an anti-Epo antibody on microtitre plates and detected by an anti-PEG antibody. A limitation of this method may be the fact that many chemical products used in everyday life (such as shampoos and toothpaste) as well as a great number of drugs[53] are PEGylated and false positive results might arise, which necessitate the confirmation of a suspicious result produced by the ELISA-based result. Hence, this test is used only as a screening method and is confirmed by IEF.

Since tests for epoetins and their derivates became common in doping control, many athletes and their medical staff appear to have resorted to the application of ESA in 'low-risk' periods during the year and subsequent storage of blood, which is used for blood doping before and during competition. Recent news coverage has shown the presence of a complex infrastructure for the harvesting, concentration and reinfusion of blood concentrates in several countries. Since autologous blood transfusions cannot be detected directly, in some sports 'biological passports' have been introduced to detect blood manipulations indirectly. Parameters such as total haemoglobin mass and reticulocytes are recorded. According to confessions by athletes, dopers try to avoid detection by tight control of haemoglobin values and injection of undetectable doses of epoetins to avoid suppression of reticulocytes by infusions of red blood cells.

Hematide and HIF-stabilizers cannot currently be detected. From a technical point of view it shouldn't pose too many problems to develop techniques for the detection of these molecules given the chemical nature of the substances. The small molecular size of these substances means that they should be readily filtered by the kidney. In the meantime an indirect sign of the use of Hematide could be missing detection of endogenous Epo in IEF. Unfortunately developers usually hesitate to provide samples to antidoping labs before officially introducing substances to the market. This gives dopers a head start over doping control.

Conclusion

Raising of haemoglobin levels is one of the most potent measures for gaining an advantage in endurance sport. Dopers have always been in hot pursuit of medical progress in the field of ESA. Every new compound poses new technical problems in doping analytics. With new drugs on the horizon, doping analysis is facing new challenges. Early support by pharmaceutical companies would help doping analysts to gain an advantage over dopers. Unfortunately this support is not always given.

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