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Chapter 13

DEVELOPMENT OF NON-ERYTHROPOIETIC ERYTHROPOIETIN VARIANTS FOR NEUROPROTECTION

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Abstract: Erythropoietin is well known to possess erythropoietic activity, but also tissue protection. Here, we present examples on how to separate these two activities. One possibility is to generate a short-lived variant by removal of EPO's sialic acid residues. Although asialo-EPO has a high affinity for the classical EPO-R, it lacks hematopoetic activity in vivo upon bolus injection, because of its short plasma half-life. Another approach we followed was to generate carbamylated EPO (CEPO), which has no affinity for the EPO receptor, but the same neuroprotective potency as EPO. Our data suggest that the cytoprotective signal transduction of EPO is distinct from the hematopoietic signaling machinery.

Key words: Asialo-EPO, carbamylation, chemical modification, mutation, neuroprotection,

1. INTRODUCTION

Erythropoietin is a 30-35kDalton glycoprotein containing about 40% carbohydrates. Blood-borne EPO is primarily produced in the kidney and controls the proliferation and differentiation of red blood cells in the bone marrow. EPO induces hematopoiesis by binding to the classical homodimer EPO receptor. EPO has two independent binding sites for the EPO receptor - one high affinity (Kd in nM range) and one low affinity (Kd in µM range) (Philo et al., 1996). The regulation of erythropoiesis by EPO is well established. However, the biological function of EPO is not limited to hematopoiesis. EPO and EPO-R are also expressed in other tissues, such as

the brain. Through the 1990's evidence accumulated that in addition to stimulating red blood cell production EPO was able to protect neurons from dying by inhibiting apoptosis and providing trophic support. In 2000, Brines et al. reported that EPO, administrated peripherally, crossed the blood brain barrier under non-pathological conditions (Brines et al., 2000). A single intraperitoneal dose of 5000 U/kg was sufficient to ameliorate disease progression in different pathological animal models.

EPO is generally considered a safe and well-tolerated treatment of anemia. In terms of using EPO as a tissue protectant agent in patients without anemia, the hematological effects of EPO are thus to be considered as side effects. In addition to the stimulation of erythropoiesis, EPO has been shown to affect the formation and function of platelets. In line with this, EPO receptors have been detected on rat and mouse megakaryocytes (Fraser et al., 1989). Wolf et al. showed that EPO in dogs stimulated the generation of platelets (thrombocytes) and that these thrombocytes as well as the whole population of platelets were functionally hyperreactive (Wolf et al., 1997b). In a second study, using a dog model of arterio-venous shunting the group reported that EPO was pro-thrombotic (Wolf et al., 1997a). In a clinical trial on healthy volunteers, EPO increased the platelet and endothelial activation as measured by P-selectin and cE-selectin (Stohlawetz et al., 2000). Van Geet found that EPO treatment in hemodialysis patients increased the number and function of platelets which is in line with the findings by Cases that EPO improved platelet function in uremic patients (van Geet et al., 1989; Cases et al., 1992). Beguin concluded that EPO treatment induced thrombocytosis at relatively moderate doses (Beguin, 1999). These studies suggest that EPO therapy is likely to increase the risk of thrombotic event even when EPO is administered for a short period. Therefore, it would be desirable to modify EPO to obtain a molecule devoid of erythropoietic effect, but with retained tissue protective effect. In the following sections, we will give examples of different ways to dissociate the tissue protective from the erythropoietic effect by modifying recombinant human EPO.

2. ASIALO-EPO

EPO contains three carbohydrate groups, which are N-linked, and one O-linked oligosaccaride. The galactose residues of EPO's sugar part are masked by up to 14 sialic acid residues, which are the terminal sugars of the branched carbohydrate chains. In the circulation, these sialic acids are slowly removed by different sialidases as EPO "ages", and galactose residues become the new terminal residues. This allows for binding of desialylated

EPO to galactose receptors (= asialoglycoprotein receptors) in the liver, followed by internalization and digestion by lysosomes. Complete enzymatic desialylation of EPO yields asialo-EPO, a molecule with very high clearance from the circulation with a plasma elimination half-life of 1.4 min (rat, 44ug/kg IV) (Erbayraktar et al., 2003). Since the protein backbone of EPO is not affected by the sialic acid content, desialylation does not affect the interaction of EPO/asialoEPO with receptors. However, a continuous stimulation of the EPO receptor by a ligand is mandatory for stimulation of erythropoiesis. Because of the very high elimination rate, asialo-EPO is functionally without erythropoietic effect in vivo. For neuroprotection, obviously, a short trigger of the signal transduction pathway is sufficient and therefore the neuroprotective properties are retained both in vitro and in vivo.

Table 13-1 shows in quantitative terms that the affinity of asialo-EPO to the soluble EPO receptor is unchanged as well as the hematopoietic bioactivity as seen in the EPO dependent human leukemia cell line UT-7. The in vitro neuroprotective effect of asialo-EPO is similar to EPO's as seen in P19 cells subjected to serum withdrawal and in hippocampal cell cultures subjected to NMDA toxicity.

Although the plasma half-life of asialo-EPO is too short to stimulate erythropoiesis in vivo, the neuroprotective effects are retained in various animal models of disease. Asialo-EPO offered protection to the same extent as EPO in a rat model of spinal cord injury {Erbayraktar et al., 2003}. Rats were injured by transient compression of the spinal cord and dosed with EPO, asialo-EPO or vehicle (10ug/kg IV) for the first three days followed by bi-weekly dosing. Both EPO and asialo-EPO treatment significantly improved the motor scores compared to vehicle treatment. The extensive damage and edema throughout the spinal cord was reduced to a core injury in the EPO and asialo-EPO treated groups.

In a rat model of stroke (permanent middle cerebral artery occlusion) a single dose of 5-50 µg/kg IV at 90 minutes post-injury reduced the infarct size by 50% measured at 24 h post-injury.

Despite the short half-life in plasma, asialo-EPO reached the CSF at concentrations relevant for the in vitro neuroprotection (0.5-30 pM). As an alternative way to monitor the brain penetration, asialo-EPO was radiolabeled and administered peripherally. Subsequent autoradiographic analysis revealed a specific neuronal localization similar to the pattern of radiolabeled EPO. Until a few years ago it was generally believed that large glycosylated proteins like EPO were unable to cross the blood-brain barrier. However, several groups have shown that peripherally administered EPO is in fact able to enter the brain (Brines et al., 2000; Ehrenreich et al., 2004; Grasso et al., 2002; Jumbe-Nelson, 2002; Jumbe-Nelson, 2002). The

presence of EPO-R on capillary endothelial cells might suggest a specific EPO transport mechanism. Recently, Martinez-Estrada and co-workers characterized such a specific, saturable mechanism responsible for EPO's transport across the blood-brain barrier (Martínez-Estrada et al., 2003).

Table 13-1. Comparison of different EPO variants

Modification	EPO-R IC50 (pM)	UT-7 EC50 (pM)	Hippocampal neurons (% protection)	P19 (% protection)	Predominant plasma half-life (H)
Rh-EPO	10	10-30	78 +/- 13	49 +/- 12	5.6
Asialo-EPO	14	10-30	71 +/- 15	45 +/- 15	0.023
CEPO	>10,000	>10,000	70 +/- 9	49 +/- 10	3.3
S100E-EPO	>10.000	>10.000	66 +/- 9	55 +/- 15	n.d.

3. NEUROPROTECTION VS HEMATOPOIESIS

A number of recent findings point to the possibility that action of EPOon hematopoiesis and cytoprotection may involve different signal transduction cascades (e.g. Digicaylioglu, Lipton, 2001). Masuda et al. reported that cultured rat astrocytes produced EPO and that the production was dependent on the oxygen tension. He proposed that EPO produced by astrocytes acted on the EPO receptor on neurons in a paracrine fashion (Masuda et al., 1994). Neurons indeed express EPO-R and that EPO's affinity to the neural receptor appears to be lower (10-20 nM) than the erythroid receptor affinity (100-200 pM). Sasaki and coworkers found that the EPO receptor expressed on PC12 and SN6 cells which both have neuronal characteristics has a correspondingly lowered affinity (Masuda et al., 1993). This discrepancy in receptor affinity between peripheral and neuronal tissue suggests that the neuronal EPO receptor might be different from the EPO receptor found in erythroid cells. Further experiments revealed different sizes of receptor associated proteins and receptor molar weight dependent on the cell type. The classical EPO receptor is a homodimer, but that does not exclude that heterodimers or oligomers could constitute functional EPO receptors in non-erythroid cells. It has for instance been reported that EPO can form functional receptor complexes with other cytokine receptors such as the CD131 receptor (Beta common chain) (Hanazono et al., 1995; Jubinsky et al., 1997).

The finding that EPO has a neurotrophic sequence unrelated to the binding sites to the classical EPO receptor and that this 17-mer peptide sequence is non-hematopoietic further suggests that cytoprotection and hematopoiesis are mediated via two distinct mechanisms (Campana et al., 1998).

Ideally, one would want an EPO molecule not just without functional lack of erythropoiesis in vivo, but a molecule that does not interact at all with the bone marrow EPO receptor. From the literature, it is known that a small chemical modification or a single mutation is enough to abolish the affinity to the EPO receptor (Grodberg et al., 1996). We speculated that it would be possible to engineer the EPO molecule to loose the affinity to the bone marrow EPO receptor, while retaining the cytoprotection.

4. CARBAMYLATED EPO (CEPO)

There are many possibilities to chemically engineer the EPO molecule. The lysines, arginines, tyrosines and the carboxyl groups on the EPO molecule can be chemically altered e.g. by carbamylation, amidation, trinitrophenylation or acylation. We explored in particular the possibility to carbamylate the seven lysines in the EPO molecule to generate homocitrulline residues (Leist et al., 2004). Under normal physiological conditions 0.8% of urea is converted to cyanate in human plasma. This readily reacts with EPO (Satake et al., 1990) to form partially carbamylated EPO (Mun, Golper, 2000). Partially carbamylated EPO is thus a naturally occurring form of EPO in man. Surprisingly, we found that although the fully carbamylated EPO molecule we produced (CEPO) is devoid of interaction with the classical EPO receptor the cytoprotective properties were retained (Leist et al., 2004). CEPO has at least 1000 fold less affinity to the soluble EPO receptor and likewise more than 1000 fold lower effect in stimulation of the UT-7 erythropoietic cells compared to EPO (figure 13-1). In addition, CEPO does not induce the Jak/Stat signaling seen with EPO in BaF/3 cells transfected with the EPO receptor. Moreover, CEPO is not capable of antagonizing EPO in the UT-7 cell even at concentrations 300 times higher than EPO. Despite the clear loss of EPO receptor interaction CEPO is as efficacious as EPO is hippocampal cell cultures subjected to NMDA. In P19 cells challenged by serum withdrawal CEPO was as efficacious as EPO in rescuing the cells (table 13-1).

Obviously, it is of major importance that an engineered EPO variant enters the CNS to at least the same extend as EPO. Indeed CSF levels of CEPO after intravenous dosing (44ug/kg CEPO) were comparable to the levels of EPO. Even 24 h after intravenous dosing the CSF levels were significantly above baseline. As opposed to the asialo form of EPO the carbamylation does not alter the kinetic profile substantially. The predominant plasma half-life of CEPO in rats is in the same range as EPO's (3-6 h). After subcutaneous injection the plasma concentrations was above 2 nM (the erythropoietic threshold for EPO) for more than 20 h. Thus CEPO

circulates long enough to stimulate erythropoiesis. To rule out the possibility that CEPO was stimulating erythropoiesis through a non-EPO receptor mechanism several experiments were conducted to examine the effects of CEPO on hemoglobin and hematocrit levels. Bi-weekly subcutaneous dosings with up to 500 µg/kg CEPO did not increase the hematocrit in mice over 10 weeks. In another experiment mice were subcutaneous dosed daily for 4 weeks. The mice dosed from 10 to 200 µg/kg CEPO had no increase in hemoglobin or hematocrit as opposed to a group of mice receiving 10 µg/kg EPO, which had a significant increase in hematocrit. It was further tested whether CEPO could antagonize the erythropoietic effect of EPO in vivo. Even ten-fold higher dose of CEPO (50µg/kg/day) could not antagonize EPO's (5 µg/kg/day) effect on hematocrit.

Table 13-2. Effect of EPO variants in animal models

	EPO	Asialo-EPO	CEPO
Hematopoiesis	+	÷	÷
Stroke	+	+	+
Spinal cord injury	+	+	+
EAE	+	+	+

+ = biological activity; ÷ = lack of biological activity

The data from the in vitro cell death models suggest that CEPO is at least as efficacious and potent as EPO in various animal disease models of acute and chronic neurodegeneration. This was tested in detail. In a rat model of stroke, CEPO decreased the infarct volume by 50% in a dose-range from 5-50µg/kg IV when measured 24 h after injury compared to a saline treated group. The tissue protection was retained even when the dosing was postponed to 4 h after onset of the injury. A similar broad time window of opportunity has been reported for EPO (Brines et al., 2000). The tissue protection correlated well with an ipsilateral reduction in inflammatory markers such as Interleukin-6 and Monocyte chemoattractant protein-1.

In a rat model of spinal cord injury the neurological function was examined over 42 days during chronic treatment with EPO, CEPO or saline (Leist et al., 2004). The CEPO treated group recovered fully from the injury with a neurological score significantly higher than the saline or the EPO-treated group. Delaying the CEPO treatment for 24hrs was as efficacious as when the treatment is initiated immediately after the injury. Even a delay of 72hrs significantly improved the recovery compared to saline treated rats.

Furthermore, CEPO was tested in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Leist et al., 2004). CEPO treatment 3 times per week significantly improved the neurological outcome in the mice immunized with myelin oligodendrocyte glycoprotein (MOG) to induce the EAE. Improvement in functional outcome was seen even when CEPO

treatment was commenced 4 weeks after the mice had reached a plateau in symptoms.

Lastly, CEPO was tested in a model of diabetic neuropathy induced by streptozotocin. CEPO treatment (3 times per week subcutaneously) improved the nerve dysfunction as assessed by the thermal nociceptive threshold in the hotplate test.

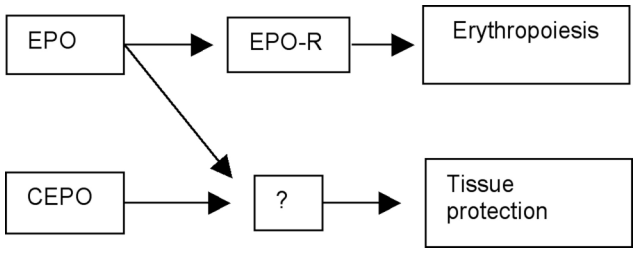

Figure 13-1. Biological activities of EPO and CEPO

5. OUTLOOK

In this chapter we have given examples of different ways to modify EPO to dissociate the erythropoietic effect from the tissue protective effect. While the principle behind asialo-EPO is dissociation based on altering the pharmacokinetic profile, the different effects of CEPO are based on altered pharmacodynamics (no interaction with the EPO receptor). Engineered proteins in clinical use differ from the natural template by altered stability, kinetic profile or antigenicity. In contrast to this, CEPO is an example of a new class of compounds with a new bioactivity profile. It has been designed to have a similar pharmacokinetic profile as EPO, but exhibiting a new mode of action at the molecular level. This model of action can best be explained by interaction with an alternative receptor transducing tissue protection signals. See figure 13-1. This receptor is unlikely to be the classical EPO receptor homodimer. One possibility is that CEPO binds a heterodimer involving only one EPO receptor. Such a receptor would constitute a new pharmacological target, and the availability of CEPO as selective ligand will allow pharmacological characterization and exploration of the underlying biology.

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