Pre-clinical criteria sustaining the safe use of recombinant human erythropoietin (EPOrh)

REVIEW

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ABSTRACT

Erythropoietin is an essential growth and viability factor to erythroid progenitors of bone marrow, regulating erythrocyte production and adapting it to the physiological needs for oxygen. Human EPO obtained through recombinant DNA (EPO-hr) technology has enabled its wide therapeutic use. Clinical experience has shown that this molecule is capable of modifying anemia associated to chronic renal failure, zidovudine based therapy in patients with Acquired Immune Deficiency Syndrome (AIDS), rheumatoid arthritis, chemotherapy, prematurity, autologous transfusions, oncohemathological diseases and others. Tissular hypoxia is the main stimulus for the synthesis of this hormone in liver and kidneys. Renal anemia can be modified in a dose dependent manner without adverse effects, disregarding the possible increase of blood pressure. Patients suffering from extra renal anemia could also benefit from the use of hr EPO. The present paper reviews the poorly disseminated preclinical findings of experiments with this hormonal growth factor, in an attempt to widen the knowledge of its therapeutic use.

Key words: erythropoietin, preclinical study, carcinogenesis, mutagenesis, toxicity

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RESUMEN

Criterios preclínicos que sustentan el uso de la eritropoyetina recombinante humana. La eritropoyetina (EPO) es un factor de crecimiento y viabilidad esencial para los progenitores eritrocitarios de la médula ósea, que regula la producción de eritrocitos y la adapta a las necesidades fisiológicas de oxígeno. La obtención de EPO humana por la tecnología del ADN recombinante (EPOhr) ha permitido su amplio uso terapéutico. La experiencia clínica ha demostrado que esta molécula es capaz de corregir la anemia asociada a diferentes estados como: insuficiencia renal crónica terapia con zidovudina en pacientes con síndrome de inmunodeficiencia adquirida (SIDA), artritis reumatoide, quimioterapia, prematuridad, tranfusiones autólogas, enfermedades oncohematológicas y otras. La hipoxia hística es el principal estímulo para la síntesis de esta hormona en los riñones y en el hígado. La anemia renal puede ser corregida independientemente de la dosis utilizada, sin importantes efectos adversos, excepto el posible incremento de la presión arterial. Los pacientes que sufren anemia extrarrenal también se pueden beneficiar del uso de la EPOhr. En el presente trabajo se describen estudios preclínicos que demuestran la seguridad y tolerancia, así como se reseñan hallazgos poco divulgados de la experimentación con este factor de crecimiento hormonal, a modo de contribución a la ampliación del conocimiento sobre su elevado índice terapéutico.

Palabras clave: eritropoyetina, estudio preclínico, carcinogénesis, mutagénesis, toxicidad

*I*ntroduction

Erythropoietin glycoprotein hormone (EPO) is a fundamental growth factor of the erythrocyte precursor cells in the bone marrow. It is produced in the kidneys and liver of adults [1, 2]. The synthesis of this hormone is triggered by tissue hypoxia in the kidneys and liver.

Endogenous and recombinant human EPO are similar with respect to their biological and chemical properties, with the exception of certain microheterogeneity in its four carbohydrate chains [3].

Renal anemia can be modified with the administration of EPOrh, in accordance with the dose, and does not cause major adverse effects, with the exception of a possible increase of blood pressure. [3]. Patients suffering from extra-renal anemia can also benefit from EPOrh. On the other hand, the formation of anti-EPOrh antibodies has been reported in a few cases in humans [3].

Initial dosage varies from 50 to 100 IU/Kg, three times a week by endovenous or subcutaneous administration (endovenously in other patients on dialysis and subcutaneous in patients). The dose must be lowered when the patient's hematocrit ranges from 30 to 33% or if the values increase by more than 4 points during a period of two weeks and it must be increased if hematocrit values do not rise in 5 to 6 points after 8 weeks of treatment [4, 5]. The dose is later personalized in order to maintain hematocrit values within the desired range (between 75 y 150 IU/Kg) [5]. The dose may be increased in patients who do not respond to the latter dose, up to a total of 300 IU/Kg. It is very unlikely that patients who do not respond at this level will actually improve with higher doses and it is therefore not recommended. The dose should be modified in 25 IU/Kg at a time [4]. The Initial dosage administered to patients suffering oncohematological disorders, anemia resulting from chemotherapy and AZT based therapy in the case of AIDS should range from100-150 IU/Kg and the maintenance dose should be personalized [6].

*T*herapeutic use and safety evaluation

Several human recombinant proteins play an important therapeutic role, especially for enhancing hematopoyesis following chemotherapy. Due to the

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fact that they are copies of endogenous proteins, it was assumed that they would be tolerated by the body. Although this assumption is valid in some cases, a preclinical safety evaluation of these proteins is necessary. Preclinical toxicity trials are of predictive importance in humans [9, 10].

During the non-clinical evaluation of different variants of EPOrh, studies are normally conducted to determine the pharmo-kinetic characteristics of the molecule, compared to a com-mercial brand, and to establish if they are innocuous [11, 12]. The most extensively used trials, which have also been thoroughly documented, are listed below:

1. Intravenous and subcutaneous pharmaco-kinetics.
2. Sole dose toxicity [13].
3. Local tolerance [14].
4. Toxicity resulting from doses administered during a period of 90 days [15].

However, among the list of non-clinical trials referenced in Part III of the Register, reporting the results of experiments conducted in animals, the trials referenced below, which are not conducted with the same frequency as the first, are considered in reports of studies carried out with this molecule. Consequently, this paper addresses aspects that are less known in preclinical studies with this recombinant hormone.

Study of the reproductive function

An assessment of the effect of the treatment with EPOrh on testicular function [16, 17] and on the physiology of the semen conducted in rats with chronic kidney failure indicated that EPOrh improves the function of both Leydig and Sertoli cells (the latter synthesize â estradiol following stimulation by the FSH hormone), which favors spermatogenesis, sperm maturation and sperm fertilizing capacity. As a result, some authors consider that the hormonal changes induced by EPOrh not only have a bearing on improving anemia but it normalizes the mecha-nism of pituitary-gonad feedback, which apparently improves the sexual performance of patients [18].

It has also been demonstrated that EPOrh can influence testicular steroidogenisis by stimulating testosterone production in males [18] without affecting the gonadotropin secretion which confirms its direct action of Leydig and Sertoli cells. In a clinical trial conducted with 8 males patients subject to hemodialysis [19] its effect on the erection of the penis and on the level of hormones secreted by the hypothalamus-pituitary-gonadal axis was demonstrated. Hence 72% of the patients exhibited an improvement of the erectile function, with no modification of their Follicle Stimulating Hormone (FSH), Lutein Hormone (LH) and zinc (Zn) levels or that of High Sensitivity Parathohormone (HS- PTH), which is an indication of the role played by EPOrh in the treatment of sexual disorders [19].

All cases reported in the literature refer to its beneficial effects and do not suggest the existence of a possible threat resulting from the administration of this hormone to humans for the treatment of the male reproductive system. Regarding its effects on the female reproductive system, reports corroborate the use of EPOrh for the treatment of gynecological disorders and ovarian pathologies without the onset of adverse reactions.

Perinatal toxicity

Following the success of clinical trials administering EPOrh for the treatment of anemia in adults with terminal kidneys disease, the reports of the first clinical trial in preterm children was published in 1990. Following that initial report, numerous assays have reported varying degrees of success in the treatment of this type of anemia. More recently, EPOrh was used during the first weeks of life for the prevention of premature anemia [20,21]. The physiology of fetal and maternal erythropoiesis during pregnancy indicates that hematopoiesis and its stimulation occur separately in both circulations [22]. Apparently, erythropoietin is the main regulator of both compartments. Human placenta is a barrier to both endogenous and recombinant EPO; consequently the cardinal precondition for the use of EPOrh for the treatment of maternal and fetal anemia during pregnancy is met. The prevalence of maternal anemia during gestation and post partum is high and therefore the use of EPOrh offers promising results as no significant adverse reaction has been reported.

The determination of erythropoietin levels in fetal blood obtained by cordocentesis (blood from the umbilical cord) and from the mother do not correlate, which indicates that EPO concentrations in the fetal and maternal compartments is regulated independently. Consequently, the administration of EPO to the mother when treating chronic anemia will not affect fetal tissues.

Possible mutagenic effect

Yajima *et al.* [23] studied the gene toxicity of EOPrh on naked mice (athymic) models. A marked increase in the frequency of micro-nuclear polychromatic erythrocytes and micronuclear reticulocytes were observed in mice inoculated with a tumor cell line trans-infected with the EPOrh gene, although chro-mosome aberrations were not observed in the spleen and bone marrow cells. The changes observed were apparently the result of the acceleration of erythroblastic maturation and proliferation induced by EPOrh. The capacity of EPOrh to induce micronuclear erythrocyte formation was assessed *in vivo* and *in vitro*. The frequency of micro nuclear reticulocytes in the peripheral blood of mice that received an intraperitoneal administration of 400-50 000 IU/Kg of EPOrh was analyzed. Samples were taken 48.72 and 96 hours following administration.

On the other hand, bacterial reversion mutation tests conducted in *Salmonella typhimurium*, TA100, TA1535, TA1537 or *Escherichia coli*, WP2 uvrA, following treatment with 188-6 000 IU/plate of EPOrh with or without S9mixture, showed small but significant increase in the frequency of micro nuclear polychromatic erythrocytes in the bone marrow of mice at doses as high as 12 500-50 000 IU/Kg of EPOrh. A clear dose/response correlation was observed, as well as a significant rise of peripheral blood micro nuclear reticulocytes, up to 96 hours after administering the preparation.

However, bacterial mutation trials fail to exhibit an increase of *Salmonella typhimurium or Escherichia colistarins* with autotrophic phenotypes after

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treating with 188-6 000 IU/plate or even at doses of 750-6 000 IU/mL. EPOrh did not induce chromosome aberrations in CHL *in vivo* or in peripheral blood lymphocytes. Chromosome aberrations were not detected in eritroide precursor cells in the bone marrow of CD-1 mice, following the admi-nistration of 100 000 IU/Kg *in vivo* [24].

Therefore, it was concluded that errors which may occur during erythrocyte enucleating or differentiation process, together with the induction of damage to the DNA or mistakes occurring during the repairing process should also be considered as possible mechanisms leading to an increase in the incidence of micronuclear cells.

Evidence seems to indicate that erythropoietin has a low mutagenic potential and apparently, the acceleration in the proliferation of progenitor cells produces errors which increase the levels of immature precursor cells in peripheral blood. However, damages to the cell genome has not been detected in the organs of animals or in bacteria.

Possible carcinogenic effect

Tumors can be induced by numerous factors such as radiation, biological agents and chemical substances of different origins. Apparently, tumor transformation is caused by changes in the cell genome or by alterations of information in the cell and its subsequent fixation and replication.

EPOrh has been used successfully in the treatment of cancer related anemia. It has also been tested in patients with tumors that exhibit platinum induced anemia. Several authors have also reported its positive effects on the oxygenation of tumors, inhibition of tumor growth and for support therapy during radiation and chemotherapy [3].

Anemia in cancer patients is associated to an the excessive production of cytokines which inhibit EPO synthesis, thus interfering with normal erythropoietin that leads to the reduction in the number of red blood cells and the body's tissular oxygenation capacity [25].

An astonishing finding is the fact that 30-60% of the mice with tumors treated daily with HPOrh for several weeks exhibited total tumor regression. When challenged again with the tumor cells, the mice rejected them. This effect was attributed to a lymphocyte T mediated mechanism and it seems that erythropoietin behaves like an anti-tumor agent [26].

The significant amount of evidence suggests that the treatment of cancer patients with EPOrh improves their survival rate and quality of life glaser *et al* [27] observed that hemoglobin levels and the administration of EPOrh were predictive factors for the response of individuals with oral and oral-pharyngeal squamous cell carcinoma to chemo and radiotherapy. Response, local control and survival rates in patients with hemoglobin values below 14.5 g/dL before treatment, and who later were treated with EPOrh were significantly higher, compared to those of patients with low values who were not treated with EPOrh.

It has also been stated that EPOrh is a good alternative to blood transfusion in the treatment of anemic patients with malignant hematological disorders and myelomas [28] and it is also recommended for maintaining hemoglobin levels in patients infected with HIV [29], who are prone to develop tumors.

However, in spite of the many observations and experiments, there is evidence in favor of a possible carcinogenic effect caused by EPOrh. The derivation of EPO signals in malignant ovarian cells results in the death *in vitro* of both these cells and of endothelial cells [30], while the injection of Anti-EPO or Anti-EPO-R soluble antibodies (erythropoietin receptor) in naked mice ovarian and uterine tumors reduce the size of the tumor. An immuno-hystochemical assay revealed the destruction of both malignant and endothelial cells with a capacity to respond to EPO.

Basal expression, and that triggered by EPO and EPO-R hypoxia in human breast cancer cells, has been reported [31], pointing to the EPO mediated stimulation of phosphorylation of tyrosine and cell proliferation. In other words, amplification of EPO signals can con-tribute to the promotion of human tumors as a result of tissular hypoxia.

Westenfelder and Baranowski [32]demonstrated that EPOrh stimulates proliferation in human renal carcinoma cells, in accordance with the dose, which in turn expressed RNAm for both the synthesis of EPO-R and the protein, while activation of the receptor stimulated their proliferation *in vitro*. Consequently, endogenous EPO or its administration when treating anemia may accelerate malignant proliferation in renal cells. However, it has not been demonstrated that these mitogenic effects also occur in patients with renal carcinoma.

In contrast, observations conducted *in vitro* with UT-7/EPO growth factor dependent human erythroleukemia cells, requires EPO for long term growth. Early gene expression such as c-fos, egrl and CIS, demonstrated, in response to EPOrh, that temporary expression of p42/44 was correlated to the equally ephemeral c-fos and egrl expression. These observation indicate that EPO-R, JAK2 and STAT5 are not necessary for proliferation initiation in these cell lines and while it was required for the sustained expression of c-fos and ergl, in other words for long term proliferation [33]. This undoubtedly tips the balance in favor of EPOrh´s inability as a tumor growth promoter. EPOrh, as a growth factor, has the potential to activate cellular mechanisms for proliferation control. Although EPOrh can stimulate tumor progression, according to results, it does not play a role in its initiation. Therefore, its paracrine and autocrine action exert a promoter effect, as opposed to its endocrine action. However, evidence against its promoter effect stem from studies conducted *in vitro* and not in biological systems, in which we have not found any report. Perhaps signals interact in the body resulting in tumor growth control.

It seems however unlikely that the plasma levels reached during the anemia treatment is actually caused by a tumor promoter impact. Concerning tumor promotion, EPO´s autocrine and paracrine production (and/or other growth factors) is of much greater importance.

In this case, exogenous administration could only affect its autocrine production as tissular hypoxia drops (this seems to be the stimulus for the endogenous production of EPO).

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Nevertheless, we consider that exogenous EPO should not have a significant impact on the production of EPO in tumors.

Toxicity in other systems

Immunotoxicity, neurotoxicity and toxicity studies are conducted to determine toxic effects on the endocrine system and especially on the immune system.

Although reports indicate that EPOrh can regulate IL-2 activity [24], its action on the endocrine system is yet to be determined as EPO receptors have not detected it in myeloid, lymphoid or monolytic cell lines [35].

As to the effects on the nervous system, it has been demonstrated that besides its hematopoeitic action, EPOrh exhibits neurotrophic properties and neuroprotector effects in hypoxic-ischemic models [35].

Neurons of the Central Nervous System exhibit EPO-receptors while astrocytes synthesize the ligand. I has been demonstrated, through RT-PCR and immunostaining that EPO-R are expressed in cultures of neurons from the hippocampus and the cortex of the brain [36]. EPOrh protects primary neuron cultures from the glutamate in the media by means of NMDA receptors (N-Methyl D-Aspartate) [37]. Some reports indicate that its neuroprotector effect may be exercised by lowering the formation of free radicals, mediated by nitric oxide or as an antagonism to its effects. Preclinical findings suggest that EPOrh may be used for treating cerebral infarctions, cranial traumas and epilepsy although further studies are necessary in order to confirm the extent of these promising observations in animal models. An important finding reinforced hope by indicating that disorders of the Central Nervous System (SNC) may be treated through the systemic administration of this growth factor.

A joint research identified EPO receptor expression in capillaries of the human brain, as well as a receptor mediated mechanism for the transportation of EPO through the hematoencephalic barrier following an intraperitoneal injection of a sole dose of the preparation in rodents and the subsequent protection against several types of neuronal damage. For example, the administration of EPO 24 hours before or up to 6 hours following focal ischemia significantly lowered the extent of infarction EPO-rh and also reduced cerebral damage due to concussion, kainite induced convulsive activity and seizures due to autoimmune encephalomyelitis [38].

In general, the neuroprotective properties expected for EPOrh are based on the fact that:

1. Endogenous EPO is expressed by the Central Nervous System.
2. Endogenous EPO can be induced by hypoxia.
3. EPO´s marked neuro-protector effect has been observed in cell cultures and in animal models.
4. EPO has multiple protective effects (antiapoeitic, neurotrophic, antioxidant, angiogenic) and
5. EPO is extremely well tolerated [39].

It also exerts an action of the neuroendocrine system.

Patients who require chronic hemodialysis present abnormal functional values of the hypothalamus-hypophysis, thyroid and gonads, including a delayed response to Thyroid Stimulating Hormone (TSH) , Thyrotropin Releasing Hormone (TRH), hyperprolactinemia, high LH levels, a marked response to Gonoadotropin Releasing Hormone (GnRH) and a low FSH secretion response to GnRH.

Following the correction of anemia (based on the treatment with exogenous erythropoietin), normal TSH and TRH responses were observed, including that of Basal Growth Hormone (GH) normal Prolactin levels and FSH response to GnRH, which may be due to improved oxygenation or to EOPrh´s trophic effects [40] which probably suggests the need for adequate oxygenation for the syntheses *de novo* of hormone proteins and/or participation of EOPOrh in hormonal function regulatory pathways.

Therefore, none of the three systems (immune, nervous and endocrine) exhibit effects other than those that contribute to the maintenance, protection and normalization of body functions. The information presented in this paper regarding the effects caused by the administration of EPOrh on organ systems and tissues that are specific of experimental animals and *in vitro* systems, together with the classical regulatory toxicology studies conducted, offers thorough understanding, which points to a good tolerance to this molecule, thus confirming the benefits expected from its use.

Conclusions

EPOrh toxicological studies confirm the tolerance and absence of toxicity of this hormone in the experimental systems evaluated. These findings were corroborated by the information presented in the reports of studies conducted on systems such as the reproductive, endocrine, central nervous system, etc and most of the researchers agree that it is safe to administer this preparation.

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