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Post-translational modification of a chimeric EPO-Fc hormone is more important than its molecular size in defining its *in vivo* hematopoietic activity

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ABSTRACT

Background: Recombinant erythropoietin (EPO) has been marketed as biopharmaceutical for anemia and chronic renal failure. Long-acting EPO variants that aimed at achieving less frequent dosing have been generated, either by the addition of glycosylation sites or increasing its molecular weight.
Methods: The hEPO cDNA linked to the human IgG Fc fragment was cloned as a single codifying gene on the pAdtrack-CMV vector, yielding the recombinant adenoviral genome. For *in vitro* and *in vivo* expression assays cervical cancer cell line (SiHa) and nulliparous goats were used, respectively. The hematopoietic activity of EPO-Fc, expressed as the differential increment of hematocrit was evaluated in B6D2F1 mice. NP-HPLC of the 2AB-labeled N-glycan was carried out to profile analysis.
Results: The direct transduction of mammary secretory cells with adenoviral vector is a robust methodology to obtain high levels of EPO of up to 3.5 mg/mL in goat's milk. SiHa-derived EPO-Fc showed significant improvement in hematopoietic activity compared to the commercial hEPO counterpart or with the homologous milk-derived EPO-Fc. The role of the molecular weight seemed to be important in enhancing the hematopoietic activity of SiHa-derived EPO-Fc. However, the lack of sialylated multi-antennary glycosylation profile in milk-derived EPO-Fc resulted in lower biological activity.
Conclusions: The low content of tri- or tetra-antennary sialylated N-glycans linked to the chimeric EPO-Fc hormone, expressed in the goat mammary gland epithelial cells, defined its *in vivo* hematopoietic activity.
General significance: The sialylated N-glycan content plays a more significant role in the *in vivo* biological activity of hEPO than its increased molecular weight.

1. Introduction

Human erythropoietin (hEPO) is a glycoprotein hormone that stimulates erythropoiesis by binding to its receptor located on erythroid progenitor cells [1]. In healthy human adults, EPO is produced in the kidney, and acts on the bone marrow to increase the production of red blood cells [2]. Patients who fail to synthesize EPO suffer from anemia and require exogenous EPO. This chronic condition is present in patients with renal dysfunction. Recombinant human EPO produced in mammalian cells have become available in recent years. However, its *in vivo* hematopoietic activity seems to be dependent on its method of production [3].

Many of the recombinant EPO in the market are expressed in Chinese hamster ovary (CHO) cells, but other cell lines, such as those derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells have also gained regulatory approval for this purpose [4]. However, given that glycosylation is a normal cellular process, the host cell line used for the production of biopharmaceuticals such as EPO has to be able to produce the glycoprotein with the desired glycan structures [5]. Functional analyses of glycosylation-related-genes on the sialylation of four commonly used mammalian cell lines showed that sialylated recombinant EPOs produced in BHK-21 and CHO cells are more effective [6]. However, these production processes are very expensive and technically demanding [7].

Several attempts had been made to overcome this technical limitation. With the objective of developing a low-cost production process, the possibility of expressing recombinant human EPO in the milk of transgenic mammals has been widely explored [8–11]. However,

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although the recombinant protein could be detected in the milk of transgenic offsprings, the expression levels were very low. In addition, severe polycythemia was observed in founder animals, probably due to ectopic expression of the gene [11].

Direct transduction of secretory mammalian cells with adenovirus vectors has demonstrated the possibility of inducing high levels of the human growth hormone in the milk of mice and goats [12]. Mammalian goat epithelium transduced with an adenoviral vector carrying the hEPO cDNA resulted in the expression of the recombinant glycoprotein at levels of up to 2 g/L [13]. This result demonstrated the ability to produce high levels of recombinant proteins in milk. However the milk-derived hEPO displayed a low *in vivo* hematopoietic activity due to its poor sialylation level [14].

Native EPO contains three *N*-glycosylation sites at Asn-24, 38 and 83 and one *O*-glycosylation site at Ser-126. It is well documented that carbohydrates play an important role in glycoprotein biosynthesis and secretion [15,16], increasing its *in vivo* half live [17,18] and improving its stability [19]. Several efforts have been successful in modifying the native structure of EPO to improve its therapeutic properties [20,21]. Approach like glycoengineering has been employed to change the carbohydrate moiety to modify the pharmacokinetic properties of EPO. The darbepoetin alfa (DA), a hyperglycosylated analogue of hEPO containing two additional N-linked carbohydrates, resulted in a three-fold increase in serum half-life and *in vivo* hematopoietic activity, compared to the unmodified recombinant EPO [17].

On the other hand, many studies have demonstrated the correlation between erythropoietin molecular size and the rate of clearance by glomerular filtration as well as by other mechanisms [22,23]. These observations suggest that increasing the molecular size of the native human EPO could lead to enhanced serum half-life. For instance, polyethylene glycol conjugated (PEGylated) EPO has a much higher molecular weight and is protected from being cleared and consequently has a longer plasma half-life. However, PEGylated conjugates lose their *in vitro* bioactivity compared to the unmodified EPO [24]. Homodimers of two EPO molecules have been obtained to increase the molecular weight of the glycoprotein [25,26]. Another strategy to modulate the biological activity of the glycohormone has been the fusion of the therapeutic protein to the Fc region of an antibody [27,28].

In the present study, we report the close relationship between the in vivo biological activity and glycosylation processing of recombinant human EPO, which is dependent on the expression system used in its production process. Our results confirm that the direct transduction of the mammary secretory cells with adenoviral vector is a robust methodology to obtain high levels (up to 3.5 mg/mL) of the chimeric EPO variant (EPO-Fc) while expression of this chimeric glycoprotein in a mammalian cell culture system enhanced its *in vivo* biological activity compared to a commercial counterpart. However, the hematopoietic activity was significant lower when the EPO-Fc was expressed in goat mammary gland. This low biological activity was related to the low sialylation of the glycan chains linked on the milk-derived hEPO-Fc. The glycan population of mammalian cell-derived EPO-Fc showed a relatively higher abundance of multi-sialylated complex structures (34%), while, neither the tetra-sialylated nor tri-sialylated glycoforms were synthesized in the milk-derived EPO-Fc chimeric hormone. Monosialylated glycans were the major charged structures linked to the EPO-Fc obtained via the adenoviral-transduced goat mammary epithelial cells. This result strongly suggests that the carbohydrate content is more important in influencing the *in vivo* half-life of EPO than the molecular weight of the recombinant protein.

2. Materials and methods

2.1. Cell lines and animals

The human embryonic kidney cell line (HEK-293A, ATCC CRL 1573) was used for the generation, amplification and titration of recombinant adenoviral vectors derived from human adenovirus serotype five (Ad5). The cervical cancer human cell line, SiHa (ATCC HTB-35) was used for recombinant expression of soluble EPO-Fc through adenoviral transduction assays. Mice (*Mus musculus* strain B6D2F1) were used to determine the *in vivo* hematopoietic activity of the recombinant chimeric protein and goats (*Capra hircus* Saanen) were used for the *in vivo* recombinant EPO-Fc expression. The use of animals has been approved by the Animals Ethics Committee of Universidad de Concepción.

2.2. Recombinant adenovirus

The replication defective adenovirus vector AdhEPO was generated using the AdEasy adenoviral vector system [29]. The hEPO cDNA linked to the 510 bp human IgG Fc fragment was cloned as a single codifying gene of 1209 bp on the pAdtrack-CMV vector. The resulting plasmid was recombined with the pAdEasy vector, which contain the Ad5 genome deleted for E1 and E3 regions. The obtained plasmid, pAdhEPO-Fc, holds the recombinant adenoviral genome.

Primary viral stocks were prepared by transient transfection of the AdhEPO-Fc genome into host HEK-293A. Adenovirus vector stocks were further amplified in HEK-293A cells, purified by a double cesium chloride gradient, titred by GFP expression on semi-confluent HEK-293 cells and expressed as gene transfer units (GTU).

2.3. In vitro expression assay

Cervical cancer cell line (SiHa) was cultured in a 75 cm^2 flasks to obtain cell confluence of 95%, using DMEM supplemented with 10% fetal bovine serum. The cells were transduced with the AdhEPO-Fc vector at a multiplicity of infection (MOI) of 25. Eight hours later the medium was replaced with fresh medium without serum, and the transduced cells were cultured for an additional 72 h. The medium was then harvested, centrifuged at 2000 *g* for 10 min and the supernatant was assayed for recombinant EPO-Fc content.

2.4. In vivo expression assay

Three nulliparous 1-year-old Saanen goats were subjected to a 2-week hormonal regimen to induce lactation [30]. The adenoviral infusion was achieved as previously described [31]. Briefly, the adenoviral vector AdEPO-Fc was infused at a concentration of 1×10^9 GTU/mL. Milk collection started 48 h after adenovirus inoculation and it was considered as the first day of milking. Milk was collected daily for 11 days and stored at −20 °C.

2.5. EPO-Fc purification from the culture medium

Purification of the EPO-Fc protein was performed by affinity chromatography using Protein-A Sepharose column activated with cyanogen bromide (Sigma, USA). The harvested culture medium expressing EPO-Fc was clarified by centrifugation and filtered through a 0.2 μm membrane. The filtrate was diluted 1:3 in 20 mM Tris–HCl, 0.25 M NaCl, pH 7.4, and applied onto a Sepharose column previously equilibrated in the same buffer. The EPO-Fc glycoprotein was eluted in 0.1 M glycine, 0.25 M NaCl, pH 3.0. Protein elution was monitored by UV absorption at 280 nm, the eluted fractions were neutralized with 100 μL of 1 M Tris–HCl, pH 8.0 and subsequently dialyzed in 20 mM Tris–HCl pH 7.4. The sample was stored at −20 °C for SDS-PAGE and Western blot analysis.

2.6. EPO-Fc purification from milk

EPO-Fc was purified from skimmed goat milk corresponding to the milking days 2–12. The milk was diluted five-fold in 10 mM Tris–HCl pH 7.4 containing 10 mM $CaCl_2$. The precipitate materials were removed by centrifugation at 9520 g for 30 min. The supernatant was