"Rapid Screening (and Confirmatory) Method for rEPO and NESP Based on Immunorecognition of its Exogenous *N*-Glycolyl-Neuraminic Content (GLEPOLYL)"

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Project Overview

The detection of recombinant EPO (rEPO) and analogues (i.e. NESP) has shown to be very challenging due to different factors. First because they are virtually identical to their endogenous counterparts. The only alleged differences so far seem to be localised in their carbohydrates. Second because they are present in urine in very low concentrations (ca. < 1 pM). Third because, as glycoproteins they are not pure single chemical entities but present as a plethora of so called isoforms. Thus each "detectable" isoform is present in much lower concentrations (i.e. < 10 fM).
The current method (isoelectric focusing, IEF) used is based on the differences observed in the charge of those isoforms. Unfortunately so far those differences do not allow an absolute identification of the recombinant species since endogenous and recombinant species seem to show just differences in the proportion in which each isoform is expressed rather than expressing new ones. Furthermore, the method is not amenable for screening purposes since it is expensive, labour intensive and very time consuming. Our group recently showed both in NESP as well as in rEPO the presence of the non-human carbohydrate *N*-glycolylneuraminic acid (Neu5Gc). It is synthesised by CHO (and BHK) cells where the recombinant molecules are produced. Unfortunately, such molecule will only be present in ca. 10% of the rEPO or NESP molecules which poses additional difficulties for their detection.

As a result, the aim of the present project is to produce a monoclonal antibody able to recognise the *N*-glycolylneuraminic acid present in recombinant glycoproteins and develop an immunoaffinity test (e.g. ELISA) that will result in a fast and cheap screening method to recognise the presence of the Neu5Gc moiety in the EPO or NESP molecules in order to determine absolutely their exogenous origin.

Results and Conclusions

The search for structural differences between endogenous erythropoietin (EPO) and synthetic recombinant biosimilars has brought to the evidence that those differences are in the carbohydrates and that the most unambiguous one is the presence of the monosaccharide N-glycolyl-neuraminic (NeuGc) as this cannot be produced by humans.

The main objective of the present project was the development of monoclonal antibodies able to selectively recognise Neu5Gc as a tool for the differentiation between exogenous and endogenously produced EPO. Those antibodies would ultimately allow the development of cheap assays (e.g. ELISA) applicable to all doping control samples.

The antigen to be used was the trisaccharide present in rEPO that contains Neu5Gc (i.e. α-Neu5Gc-(2→3)-β-D-Galp-(1→4)-β-D-GlcpNAc) as a control, the same trisaccharide containing Neu5Ac, the most abundant structure and present in all EPOs was also needed.

After a great effort to obtain those compounds using a chemical synthesis, it became evident that a new approach had to be followed. A bioenzymatic approach was applied and those structures were successfully synthesised at last.

Standard procedures were followed to develop monoclonal antibodies by immunizing mice with the antigen conjugated to KLH through a chemical bridge used as spacer. However, neither by our own lab nor by specialised contract companies dedicated to the production of antibodies, it was possible to obtain stable hybridomes that produce antibodies able to differentiate between the antigen and the control trisaccharide.

One last attempt was made using the phage display methodology. This approach would not depend on the immunogenicity of the trisaccharide but on the ability to select the proper recombinant antibody from a very large library of phages expressing them.

After an initial selection of hundreds of potential candidates, further expression and selection of the most selective clones led us to choose two clone candidates called B10 and D1. The first clone (B10) showed to have a higher affinity for rEPO alfa or beta, expressed in CHO cells than for Dynepo, expressed in human cells, thus not containing Neu5Gc. Conversely, clone D1 showed a clear preference for Dynepo with respect to rEPO alfa or beta.

However, those findings needed to be checked against endogenous EPO and further optimised to prove the selectivity to make them useful in any of the potential analytical methodologies applicable.

PUBLICATIONS/PRESENTATIONS RELATED TO THE PROJECT

- Purification of Erythropoietin from human plasma samples using an immunoaffinity well plate. J. Mallorquí, E. Llop, C. de Bolòs, R. Gutiérrez-Gallego, J. Segura and J.A. Pascual . J Chromatogr B. 2010; 878: 2117-2122.

- Potential for the differential elution of EPO from immunoaffinity kits to distinguish endogenous from exogenous EPOs. Presented to the Manfred Donike Workshop. 27th Cologne workshop on Dope Analysis. 1-6 March 2009.

- New screening protocol for recombinant human erythropoietins based on differential elution after immunoaffinity purification. J. Mallorquí, R. Gutiérrez-Gallego, J. Segura, C. de Bolòs and J.A. Pascual. J Pharm Biomed Anal 2010; 51: 255-259.