Project Review

"Structural elucidation of the glycans present in human urinary EPO"

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The current doping control method to differentiate endogenous EPO from its recombinant analogues (rhEPO or NESP) is based on the separation of the different chemical entities (microheterogeneity) based on their isoelectric point (which in other words means their charge at a particular pH).

The isoelectric focusing (IEF) behaviour of those substances has showed two important features:
1.- The differences are located in the carbohydrate moiety.
2.- There must by a number of negatively charged groups, additional to the sialic acid content, present in the endogenous EPO.

Since at present we do not know exactly what we are looking at when an IF analysis is performed, the structural elucidation of the carbohydrate moiety of those molecules, responsible for their analytical behaviour, is essential in the legal field of doping control. Many approaches have been followed in order to elucidate the glycan structures of pure recombinant standards (rhEPO and NESP) but the key issue of the endogenous substance (uhEPO) has not been solved. Our group has already studied and elucidated the glycan structures present in rEPO (BRP standard) both as a whole and on discrete IEF separated bands as part of a research project awarded by the WADA (acronym: RHEPOSE). The structural elucidation of the chemical species present in each IEF band has been found as well as the presence of N-glycolyl-neuraminic acid in both rEPO and NESP. However, so far endogenous urinary EPO has posed additional difficulties to the entire scientific community for its elucidation.

The endogenous urinary EPO standard used (NIBSC 2nd international reference preparation) has shown to fit well with the isoelectric profile of real urine samples in the current doping control method. Hence it is a very good starting point for the investigation of those glycan structures responsible for their analytical behaviour. This uhEPO standard is a concentrate from real human urine and as such, a mixture of many endogenous components, most of them at much higher concentrations than EPO. A highly selective purification procedure is an essential first step in order to approach any further analysis.

The aims of the present project are:
- To develop a purification procedure for endogenous urinary EPO.
- To perform structural elucidation of the EPO released glycans based on:
    - · Mass spectrometric analysis (MALDI-TOF, ESI-Q-TOF)
    - · Monosaccharide analysis (GC/MS)
    - · Sialic acid speciation (reverse phase HPLC)
    - · Weak anion exchange (WAX) profiles
    - · Normal phase LC profiling
    - · RAAM (reagent array analysis method)

“Structural elucidation of the glycans present in human urinary EPO. (CHARGEPO)”

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Results and Conclusions

The charge differences between urinary EPO (uEPO) and its recombinant analogues (rEPO) are the basis for the current doping control method (isoelectric focusing or IEF). Previous studies have shown that those differences are located in the carbohydrate moiety of the glycoprotein. Surprisingly, uEPO shows a behaviour compatible with the presence of additional charges, other than the maximum number of sialic acids located in the terminal positions of the glycan antennae. The elucidation of the structures, unique for the endogenously produced EPO, is essential firstly to understand what we are observing and secondly as the basis for future unequivocal tests. The aim of the present project was the elucidation of the glycan structures of uEPO, first developing an appropriate immunoaffinity purification procedure to isolate the protein and then applying an array of glycan analysis methods for their structure elucidation. Immunoaffinity purification was achieved by using an agarose based column (Affi-gel HZ) to which a monoclonal antibody (clone 9C21d11 from R&D systems) was linked through the carbohydrates of its Fc region. This linkage ensured the appropriate orientation of the antibodies and avoided prior problems found when other approaches (e.g. CNBr activated sepharose) were used. Given the low amounts of uEPO present in urine, the project started focusing the purification on the use of the uEPO standard from NIBSC. A total of 3.1 µg of uEPO were purified using that kind of column with an overall recovery of 59%. The final 1.8 µg of immunopurified uEPO were further purified by separation in an SDS-PAGE gel. The band co-migrating with an rEPO standard used as reference (as well as the neighbouring bands) were excised from the gel and further processed for:

-protein identity confirmation

-weak anion exchange (WAX) HPLC analysis of the released glycans (charge analysis)

-MALDI-TOF MS analysis for structure elucidation.

Results show that despite the extensive purification, there were other proteins present in the uEPO fraction. Therefore, the extent of their contribution to the glycan analysis results is unknown. Glycan analysis both by WAX HPLC and MS gave consistent results with two major groups of structures, identified as oligomannose (neutral) and di-antenary di¬sialylated (di-charged). These major structures do not justify the IEF behaviour of uEPO, on the contrary they seem to show less charge than the corresponding rEPO products. No other structure justifying additional charges could be found. Further efforts are needed to extensively purify larger amounts of uEPO together with the application of techniques minimizing the potential losses of labile structures in order to fully understand all this hidden aspects of uEPO.

- Purification of EPO from real urine is also considered in a later stage, in order to check that the uhEPO standard us structurally representative of what can be found in the urine of healthy human beings.

The information obtained will be used to develop a method to differentiate between endogenous and recombinant molecules based on their respective unique carbohydrate structures.