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Assessing the charge heterogeneity of different erythropoietins

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INTRODUCTION

The use or abuse of erythropoietin in various sport disciplines is a well-known phenomenon that persists despite the tremendous effort to catch the cheats. The single best method, capable of differentiating between endogenous and exogenous erythropoietin was developed by Lasne *et al*¹⁻³. In a comparative isoelectric focussing (IEF) profile, recombinant EPO (rEPO) from chinese hamster ovary (CHO) cells (rEPO from the European Pharmacopeia) displays 4-6 bands at pI-values around 4.5-5.0. Darbepoietin (NESP) shows 4 discrete bands at pI-values of 2.9-3.2 and endogenous urinary erythropoietin (uEPO) displays more than 10 isoforms covering the pI-range from 2.9 to 4.5. However, up to today no solid explanation has been provided for the origin of the different IEF-isoforms. Erythropoietin (EPO) is a glycoprotein hormone containing a single polypeptide chain of 165 amino acids, contains two disulfide bonds (7-161 and 29-33), four oligosaccharide chains: three Asn-linked sugars (Asn24, Asn38, Asn83) and one mucin-type sugar (Ser126)⁴. Since the glycosylation of the protein is a post-translational process influenced by the type of cell in which the protein is synthesised, different variants in the carbohydrate moieties are synthesised as a function of the glycosyltransferase presence in the cell.

We hypothesise therefore that the different IEF profiles observed for rEPO, NESP, and uEPO should stem from different glycosylation profiles. Here we present the preliminary results of an enzymatic study aimed at the clarification of the IEF profiles of rEPO, NESP and uEPO.

EXPERIMENTAL

Materials

Biological reference product (BRP) of rEPO was obtained from European Pharmacopoeia (Strasbourg, France). NESP was purchased as pharmaceutical preparation from Amgen (Breda, Holland). Human urinary erythropoietin standard was obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, United Kingdom). Aryl sulfatase (*Helix pomatia*) and glucuronidase (*Escherichia coli*) were obtained from Sigma (Steinheim, Germany). Neuraminidase (*Arthrobacter ureafaciens* recombinant *E. coli*), N-Glycosidase (PNGase F) from *Chryseobacterium meningosepticum* and phosphatase (*E. coli*) were purchased from Calbiochem® (San Diego, United States). All other chemicals were of the highest purity commercially available.

Digestions

Standard digestions were performed in a total volume of 20 µl using approximately 2 ng of glycoproteins. In the case of individual sulfatase, glucuronidase, and neuraminidase digestions the buffer used was 50 mM acetate buffer (pH 5.5) and 0.3% of bovine serum albumin (BSA). In the case of complete sialidase digestions 20 mU were employed whereas in the case of a partial desialylation 20 µU of enzyme were applied. For PNGase F digestions the buffer was 50 mM Tris-HCl, 50 mM EDTA, 1% β-mercaptoethanol and 3% SDS (pH 7.45) and for the phosphatase was used 50 mM Tris-HCl, 5 mM DTT, 200 µM MnCl₂, 100 µM EDTA and 200 µg/ml BSA (pH 7.0). In case of multiple enzyme digestion, the incubation was adjusted to the most demanding conditions. All the solutions were incubated over night at 37° C.

Isoelectric focusing (IEF)

Enzymatic digestions were heated at 80 °C for 3 min and applied directly to the gel. Different IEF gels were evaluated with the aim to obtain enhanced resolution in the basic or acidic area according to the possible findings. Both gel components and composition were modified as

well as the electrode composition to achieve optimal results. Evaluations of the resulting digests were performed according to Pascual *et al.*⁵

RESULTS AND DISCUSSION

Parting from the hypothesis that the origin of the IEF heterogeneity resides in the glycosylation of the different erythropoietins and analogues, the attention was focussed on the charged residues of these glycans: *N*-acetyl neuraminic acid (sialic acid). A mathematical exercise results in a maximum of 14 sialic acid residues for rEPO, corresponding to three N-linked and one O-linked chains⁶. For NESP, the total number yields 22 sialic acids as a result of two additional N-linked chains incorporated into the glycoprotein⁷. Mass spectrometric analyses of rEPO before and after chemical desialylation yielded a mass difference of ~3.2 kDa (data not shown) which equal a mean of 11 sialic acid residues for this compounds. As such the most acidic isoform in the IEF profile should correspond to the 14 calculated sialic acid residues. Having verified this feature no straightforward explanation can be provided for the IEF profile of uEPO as it contains the very same theoretical number of sialic acids as rEPO (14). As such the endogenous compound should carry additional charges of unknown origin, resulting in more acidic isoforms.

Different enzymatic digestions were performed to evaluate the contribution of different charged groups to the IEF profiles of rEPO, NESP and uEPO. The most revealing results have been summarised in two different IEF gels that will be commented. In a first gel with a pI range from 2-6 the results of digestions with glucuronidase, aryl sulfatase and neuraminidase (partial and total) are shown (figure 1). The results of enzymatic incubations are compared to the standard preparation treated in a similar way. The glucuronidase digestions did not induce any change in IEF behaviour for rEPO, NESP and uEPO. As the glucuronidase used has a renowned specificity for glucuronic acid present in steroid conjugates no apparent change was expected. As, no glucuronidase addressing galactose-attached glucuronic acids is commercially available this item could not be pursued further. Sulfatase digestions were carried out employing an aryl sulfatase, possible addressing tyrosine sulfation.

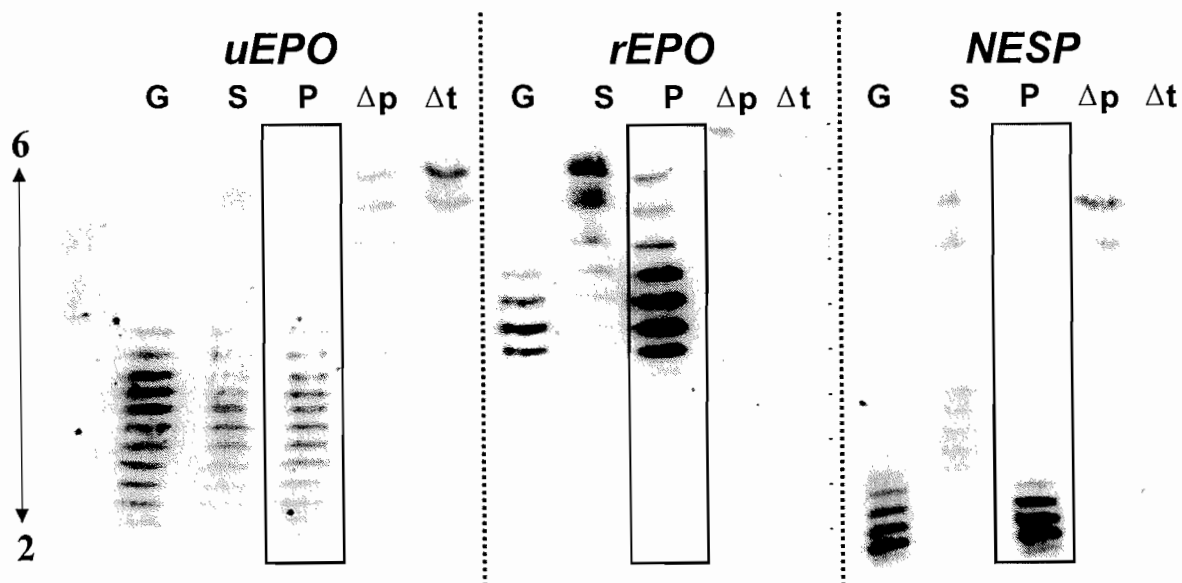


Figure 1. IEF profiles of uEPO, rEPO, NESP (from left to right and different samples are separated by a dashed line) after different enzymatic digestions. (G: Glucuronidase, S: Sulfatase, P: Standard, Δ p: Neuraminidase partial, Δ t: Neuraminidase total)

When the IEF profiles of the resulting digestions were compared to the standards only a slight movement to the basic area was observed for uEPO. In the lane of rEPO in this gel the relative displacement appeared similar to that observed for uEPO, albeit that the most intense bands appear at the top of the IEF gel. However, sulfatase-digestion of NESP induced a drastic shift to the basic area a long the gel in comparison with the standard profile suggesting the existence of a large number of sulphated groups in this molecule at that point.

Partial neuraminidase digestions showed in all the preparations a movement of the isoforms towards the basic area, caused for the gradual loss of sialic acid residues. The profile of rEPO suggests the presence of isoforms with a pI more basic than 6 for which this result cannot properly be evaluated from this experiment. When a total sialidase digestion was carried out, remarkable differences in the profile for uEPO vs. rEPO and NESP were observed. Where in the case of the recombinant molecules no single molecule could be detected, the profile of uEPO still showed at least 3 bands in the uppermost basic area. In view of these results an expanded pI range for the IEF gels was investigated. Nonetheless, in these experiments, partial neuraminidase digestion of NESP gave a remarkably similar profile as the aryl sulfatase digestion of NESP. The combined results from both the neuraminidase and sulfatase digestions of NESP suggest that both enzymatic treatments address the same charge. As the neuraminidase employed was of recombinant nature a high purity (in contrast to the sulfatase

preparation), a sialidase-type effect of the sulfatase digestion cannot be excluded. At this point it remains unclear whether this sialidase-type effect relates to a co-purified sialidase (of restricted specificity and limited activity) of the partial purified sulfatase preparation or whether it relates to a permissive active domain of the sulfatase, capable of harbouring sialic acid residues linked α 2-3 to galactose. In both cases a pronounced effect would be visualised for the recombinant molecules that contain exclusively sialic acid residues in α -2,3-positions and slight effect on the urinary variant that contains both α -2,3- and α -2,6-linked sialic acids.

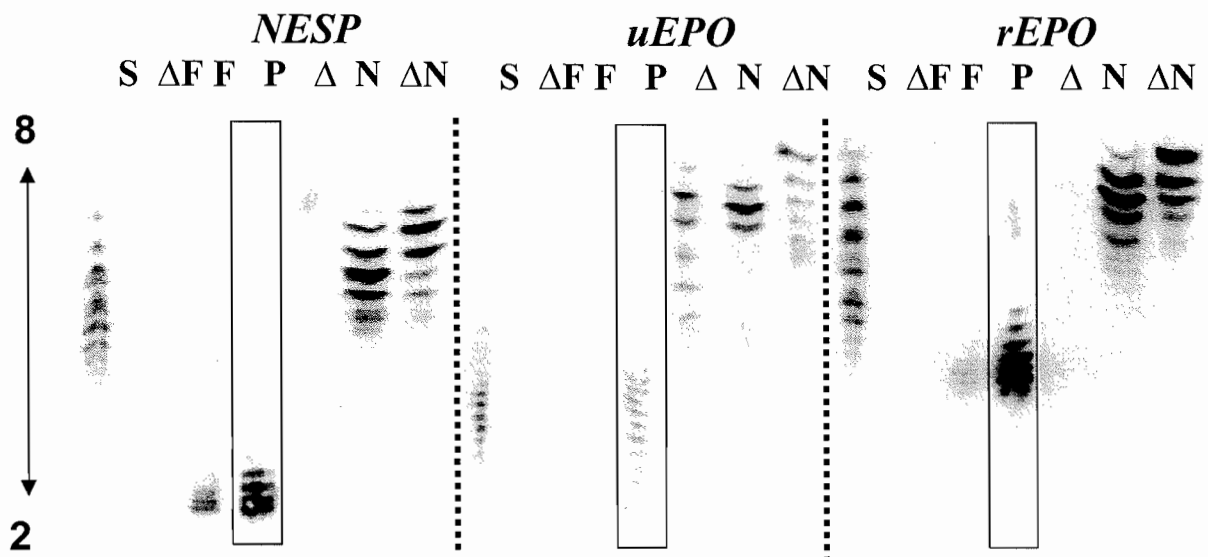


Figure 2. IEF profiles of NESP, uEPO, and rEPO (from left to right and different samples are separated by a dashed line) after different enzymatic digestions. (S: sulfatase, Δ F: Phosphatase and Neuraminidase, F: Phosphatase, P: Standard, Δ : Neuraminidase, N: PNGase F and Δ N:PNGase F and Neuraminidase)

Following many different gel compositions and components finally a gel setting was selected that permitted visualisation of a pI range from 2 to \sim 9. Figure 2 summarises the results of several digestions performed. Glucuronidase digestions were left out in view of previous results. The sulfatase digestions gave very similar results as previously observed. In this case for rEPO a similar shift as for NESP could be observed due to the extended pI range, reinforcing our previous comments on the particular activity of the sulfatase. Additional studies are being done in our laboratory to clarify this phenomenon. Nevertheless, the sulfatase digestion and the subsequent localization of the isoforms for rEPO, NESP and uEPO can be useful in a confirmatory method for unambiguous discrimination between uEPO on one hand, and rEPO and NESP on the other.

Unfortunately, in the case of the neuraminidase digestion, identical results were observed for the recombinant molecules, i.e. a basic shift outside the range of the gel. Again, uEPO presented 6 to 8 reminiscent bands shifted towards the basic area. This evidence demonstrates that endogenous erythropoietin contains charged molecules that are not present in rEPO or NESP. Furthermore, the neuraminidase digestion can be used as a tool to distinguish uEPO from rEPO and NESP.

Digestion of any sample with a ser/thr/tyr-phosphatase resulted in a nearly empty lanes. Only at prolonged exposure times faint bands could be observed at the original positions for each molecule indicating that this particular incubation (that required a specific buffer) was incompatible with the detection protocol. This observation was reinforced after performing a combined sialidase-phosphatase digestion and absence of bands for uEPO.

Finally, the localisation of the additional charges present in uEPO could be positioned in the carbohydrate moiety through specific de-N-glycosylation. Digestions with PNGase F, or the combination of PNGase F and sialidase, rendered all molecules, both recombinant and endogenous, the same. The shift in the IEF profiles when going from the single-enzyme system to the two-enzyme system corresponds to the sialic acid residue of the O-linked carbohydrate chain. The different IEF profiles observed for rEPO and NESP correspond solely to the inclusion of two additional aspartic acid residues in the latter after the digestion. A theoretical exercise gives pI values of 8.74 for N-deglycosylated rEPO and 5.53 for N-deglycosylated NESP. As PNGase F cleaves off the total N-linked glycan chain the differences, previously observed, must reside in this part of the molecules. Possibly these additional charges correspond to sulphate groups located at either the 6 position of *N*-acetyl glucosamine or the 3/6 position of the galactose as described for baby hamster kidney (BHK) cell derived N-glycans⁸. Unfortunately, no specific enzyme addressing these residues is available to corroborate this for which unequivocal structural evidence will have to come from other techniques. Finally, the PNGase F digestions corroborated the earlier findings on the peculiar behaviour of the sulfatase with respect to the recombinant molecules. After de-N-glycosylation all molecules are identical when it comes to charges for which the theory of extensive protein sulfation (*vide supra*) of the recombinant molecules cannot longer stand-up.

CONCLUSIONS

We have demonstrated that endogenous and exogenous erythropoietins or analogues are structurally different. The differences are related to the charges contained in the different kind of molecules and these charges reside in the carbohydrate moieties. However, these different charges are not due to differences in sialylation but most probably relate to sulphate groups (Gal-O3/6 and/or GlcNAc-O6) present in uEPO and absent in recombinant variants.

Employing specific enzymatic digestions the IEF profiles can be selectively modified:

- Aryl sulfatase digestion “only” affect recombinant species and do “not” affect endogenous EPO
- Sialidase digestions result in an empty profile in case of recombinant species and a basic- shifted profile for endogenous EPO.

These observations can be of great value for confirmatory protocols.

Finally, the remarkable substrate specificity of the sulfatase from *Helix pomatia* requires additional research in order to pinpoint its exact activity.

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