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GlycoStation™ 4

Technical Information

Glycotechnica

Glycan Profiling of Erythropoietin

It is well known that glycan structures of therapeutic proteins or antibody drugs affect their drug efficacy. As one of such examples, we have profiled the glycan structure of Erythropoietin (EPO) using GlycoStation. This technical report outlines the result of this experiment.

◆ The function of EPO and its application as a drug

Erythropoietin (EPO) is one of hormones produced in liver, and consists of 165 amino acids. EPO controls production of red blood cells by stimulating the growth and differentiation of erythrocytic progenitor cells as a result of its binding to the receptors on the surface of hematopoietic cells in a hematopoietic tissue. When the renal function is normal, anemia is automatically recovered by controlling EPO production level. However, in the case of anemia due to renal failure, the production of red blood cells is suppressed with the depressed EPO production, and finally results in anemia. Recombinant EPO is now widely used as a therapeutic drug to remedy anemia. One of the problems of EPO treatment is that patients need to be injected below the skin so frequently. To solve this problem, the second generation EPO which has a longer half-life has been developed by modifying the glycan structure.

◆ The second generation EPO formulation

EPO has three N-linked glycans and one O-linked glycan, and its glycan content is as high as about 40% in weight. The N-linked glycans form complex types with bi-antennary to tetra-antennary branching. Bi-antennary or tri-antennary is linked to Asn-24, and tetra-antennary is usually linked to Asn-38 and Asn-83. These tri-antennary and tetra-antennary structures have usually polylactosamine, which is an iteration structure of N-Acetyl lactosamine (Gal β 1-4GlcNAc). The O-linked glycan is tri-saccharide sialyl-T (Gal β 1-3GalNAc linked a sialic acid residue), or tetra-saccharide disialyl-T (Gal β 1-3GalNAc linked two sialic acid residues).

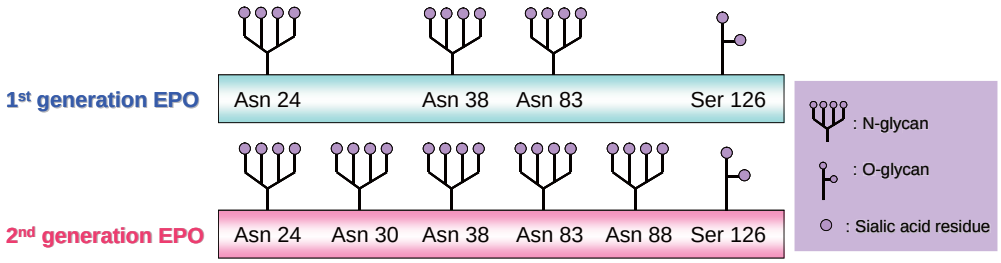

Fig. 1 Glycan structure of EPO

When EPO's terminal sialic acid residues are transected and eventually the exposed terminal galactose residues bind to galactose binding proteins expressed in liver, EPO is captured in liver and metabolized. EPO has usually about 10 terminal sialic acid residues, and thereby is protected from the metabolizing process. Therefore, while the in vitro activity of EPO gets stronger with decreasing terminal sialic acid residues due to faster binding to receptors, the in vivo activity gets stronger with increasing terminal sialic acid residues owing to longer lifetime. Based on these findings, the 2nd generation EPO has two more N-glycans with terminal sialic acid, in order to enhance hematopoietic activity with artificially prolonging the lifetime, although binding affinity to receptors gets weaker. (see Table I)

Table I Pharmaceutical and clinical characteristic for EPO

1st generation EPO		2nd generation EPO
30,400 Da	MW	37,100 Da
14	Maximum number of sialic acid side chain	22
40%	Rate of glycan	51%
High	Receptor binding affinity	Low
Short	Half-life cleared from blood	Long
Weak	Hematopoietic effect	Strong

(Reference：Pharmacological and clinical profiles of long-lasting erythropoietin（darbepoetin alfa; NESP®）, Nobuo Nagano, (Folia Pharmacol, Jpn.)131, 291 ～ 299)

◆ Glycan Profiling Analysis with LecChips

Fig.2 shows comparative glycan profiling between the 1st generation EPO and the 2nd generation EPO. It was shown that these glycan profiling patterns reflect fingerprints of N- and O-glycans expressed on EPO.

For instance, it can be estimated that tetra-antennary or tri-antennary N-glycans are highly expressed, because PHA-L (a representative lectin binding to tetra-antennary N-glycan), and ACG (a lectin binding to tetra- and tri-antennary N-glycans) show higher signal intensities. It is also considered that there may be some influence on the profiling patterns from other binding specificity of ACG, for instance which has high affinity to α2,3 sialic acid. It is also suggested that there are modified N-glycan structures by core fucose and α2,3 sialic acid, from the signals for AOL and AAL (fucose binders), and from the signal for MAL I (α2,3 sialic acid binder) without any signals for SNA, SSA, TJA I (α2,6 sialic acid binders). Signals for PHA-E, DSA, LEL, and STL (lactosamine and/or polylactosamine binders) are also detected.

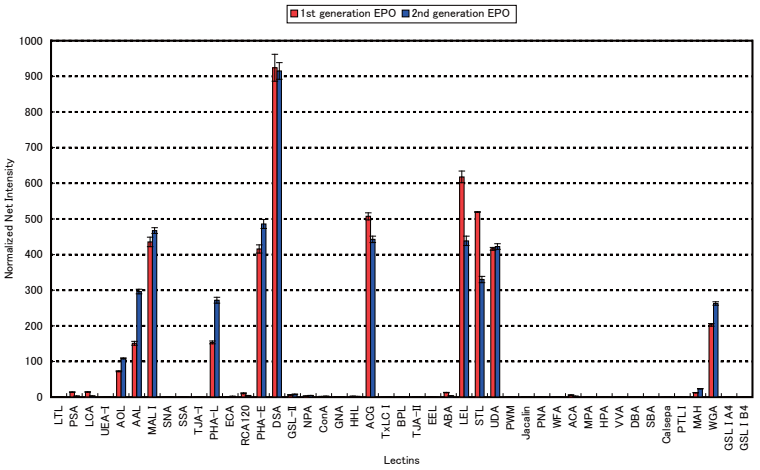

Fig. 2 Glycan comparison between 1st generation EPO and 2nd generation EPO

As for O-glycans, signals for MHA (disialyl-T binder), and ABA (sialyl-T binder) are detected. Although these signals are weaker than the other lectins, we have confirmed that the signal gets stronger with increasing sample concentration (see Fig.3). No signal for PNA (T-antigen binder) suggests that these are results reflecting disialyl-T or sialyl-T binding on Ser-126.

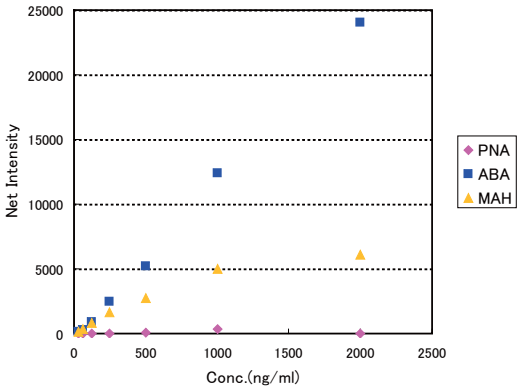

Fig. 3 Sample concentration dependence in O-glycan binder (1st generation EPO)

The difference between the 1st generation EPO and the 2nd generation EPO is the number of N-glycans per an EPO molecule. Reflecting this, the signal for PHA-L (tetra-antennary N-glycan binder) gets higher in the 2nd generation than the 1st generation. From the difference in the signals for AOL and AAL (core fucose binders), it is estimated that the expression level of core fucose is higher in the 2nd generation. (see Fig.2)

- Protocol -

1. Enrichment and Buffer Exchange
   1-1. Apply EPO onto an ultrafiltration filter[1] and centrifuge to enrich.
   1-2. Add PBS[2] to the sample and repeat centrifuge process, and recover the sample.
2. Fluorescent labeling
   2-1. Quantify protein concentration by a Micro BCA Protein Assay Reagent Kit[3]. (reaction time =2h)
   2-2. Prepare 50μg/ml, 20μl of sample with PBS, and reacts with Cy3 Mono-Reactive dye 100μg labeling[4].
   2-3. Incubate for 1h at R.T.
   2-4. Remove excess free-Cy3 with a gel filtration column[6] washed by TBS[5].
3. Measurement
   3-1. Dilute samples with Probing Solution[7] to appropriate concentrations.
   3-2. Wash LecChip™[8] three times with the Probing Solution, and then apply samples onto LecChips(100μl/well).
   3-3. Incubate LecChips over night.
   3-4. Measure fluorescence patterns without any washing of LecChips by GlycoStation™ Reader 1200[9].
   3-5. Analyze glycan profiling patterns by Array-Pro Analyzer[10] and GlycoStation™ Tools[11].

Note

1) Amicon Ultra-15, Ultracel-5K(MilliPore, #UFC900596)
2) PBS(-) pH7.3
3) Micro BCA Protein Assay Reagent Kit (PIERCE, #23235)
4) Use the equivalent amount of Cy3 Mono-Reactive dye pack (GE, #PA23011) to that used for 100μg protein labeling.
5) TBS pH7.5
6) Zeba™ Desalt Spin Columns, 0.5ml (Thermo,#89883)
7) Probing Solution (Glycotechnica)
8) LecChip™ (Glycotechnica)
9) GlycoStation™ Reader 1200 (Glycotechnica)
10) Array-Pro® Analyzer ver.4.5(MEDIA CYBERNETICS)
11) GlycoStation™ Tools (Glycotechnica)