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Review

Erythropoietin: physico- and biochemical analysis

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Abstract

A hormone, erythropoietin, mainly produced in adult kidneys and fetal livers, acts on bone marrow erythroid progenitor cells to regulate the production of erythrocyte in mammals. As a result, the oxygen carrying capacity of blood increases and the increased oxygen supply raises the cardiac function and physiological working capacity. Erythropoietin is possibly misused by athletes in sports for the purpose of improving performance. Presently there is no discernible and specific method to identify erythropoietin administration for doping control. To address this practical problem, this paper presents a summary of the applications of analytical biotechnology, especially the structural characterization of erythropoietin.

Keywords: Reviews; Erythropoietin

Contents

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1. Introduction

Doping control on athletes has been done for over 30 years. The International Olympic Committee (IOC) has banned the use of any chemical agent to better performance in sports [1]. The banned drugs, traditional doping agents, are stimulants, narcotic analgesics, anabolic steroids, β-blockers and diuretics. In addition, blood doping agents and peptides such as erythropoietin (EPO), testosterone and growth hormone (GH) were banned by the IOC starting from 1987 [2–5]. For doping control, traditional doping agents can be detected in urine samples. Peptide substances, on the other hand, can hardly be detected in such samples, but can easily be detected in blood samples [6]. Moreover, bioanalytical techniques and instrumentation have been improved to detect banned substances in urine samples over the past 30 years. New bioanalytical techniques are required to control blood doping and the misuse of the peptide hormone in the blood.

No mention is made here about other doping agents except EPO. Since EPO regulates the production of erythrocytes in mammals, it may help athletes to improve performance in sports [6–8]. Recombinant human EPO (rHuEPO) was commercially available since 1988. However, presently, there is no discernible and specific method for identifying EPO administration [9–11]. Major difficulties in identification are: (i) the presence of EPO in serum and urine at picomolar levels (130–230 pg EPO/ml of serum corresponding to 10–18 mU/ml), (ii) the similar homology between natural EPO and rHuEPO and (iii) the 5–6 h half-life of EPO in blood after injection.

How can the administration of rHuEPO be differentiated from natural EPO in blood samples? Is it possible to identify unequivocally rHuEPO administration in blood samples despite the above mentioned three major difficulties? To address these practical problems, this paper presents a summary of the applications of analytical biotechnology. Structural characterization of EPO is discussed in detail below and may initiate future study.

2. Erythropoietin

2.1. Erythropoiesis

Erythropoiesis is the process that regulates the production of erythrocyte from the blast cell. Erythropoiesis depends on the hormone called erythropoietin [7,8,12]. Erythropoiesis is the only possible way of making more erythrocytes by means of stem cells. Because of the absence of nuclei, endoplasmic reticulum, mitochondria and ribosome, erythrocytes cannot grow or divide in an adult mammal [13,14]. As shown in Fig. 1, tissue hypoxia stimulates cells in the kidneys to synthesize and secrete 1000-fold increased amounts of EPO into the blood stream. EPO, in turn, enhances the production of more erythrocytes until the level of tissue oxygenation reaches normal levels. Thus, erythropoiesis is a typical negative feedback control system.

2.2. Physiological and biochemical properties of EPO

The hormone EPO, mainly produced in adult kidneys and fetal livers, acts on bone marrow erythroid progenitor cells to regulate the production of erythrocytes in mammals [7,14,15]. Generally, the

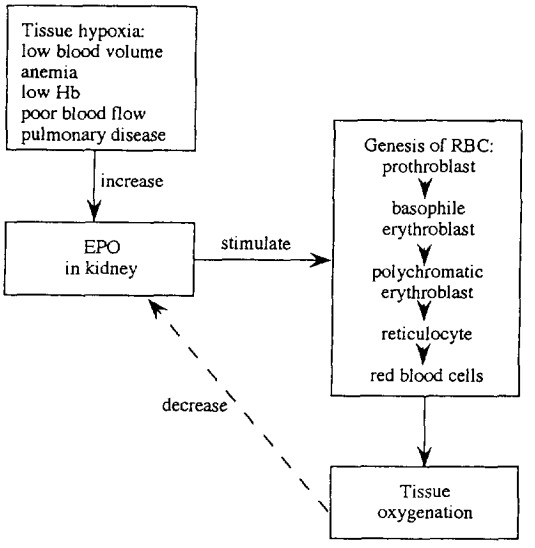

Fig. 1. Schematic diagram for the genesis of red blood cells.

synthesis of EPO, stimulated by low oxygen pressure (hypoxia, which is caused by low blood volume, anemia, low hemoglobin, poor blood flow and/or pulmonary disease), forms new erythrocytes in the kidneys. As a result, the oxygen carrying capacity of blood increases and elevates the value of hemoglobin (Hb), hematocrit (Hct) and transferrin soluble receptors (Tfr) in the blood stream. Therefore, EPO can correct renal anemia and can be used as a transfusion substitute [16–20]. To identify EPO administration, the erythrocyte morphology and biochemistry and the serum concentration of the receptor for transferrin should be checked. As shown in Table 1, characteristics of blood for a normal person are as follows: the concentration of Hb is 14–16 g/dl of serum, Htc is 40–45% of blood, the number of red blood cells (RBC) is 5 million/mm^3 and the concentration of EPO is 130–230 pg/ml of serum (corresponds to 10–18 mU/ml). The state where the four above characteristics are lower than normal is termed anemia, whereas the state where the four characteristics are higher than normal is termed polycythemia. To cure renal anemia, 30 U EPO/kg of body weight has to be taken every other day for 30–45 days. Thus, the amount of EPO hormone required for a year of treatment is of the order of milligrams. However, the increased oxygen supply can also raise the mammalian cardiac function and physiological working capacity, such that EPO can improve the physical performance of athletes in aerobic sports.

EPO is one polypeptide chain of 166 amino acids (p*I* 4.5–5.0) with two disulfide bonds (between 7 and 161, between 29 and 33) and four polysaccharide chains (three *N*-linked at Asn24, Asn38, Asn83, and one *O*-linked at Ser126) as shown in Fig. 2 [7,14,15,21]. The molecular activity of EPO is 30 000–34 000 Da, but the molecular mass of the peptide chain only is about 18 000 Da [22–26]. Thus, about 40% of the fully glycosylated EPO consists of carbohydrate. Carbohydrate plays an important role in the biological activity of EPO [27–34]. Since EPO is produced by the kidneys and is targeted to bone marrow, it should be protected against hepatic clearance during blood circulation [35–37]. The terminal sialic acids of EPO are required for the EPO hormone to reach target sites. These terminal sialic acids prevent rapid clearance by hepatic receptors which recognize exposed galactose residues [33,38–40]. Thus, the in vivo activity of EPO depends on the number of sialic acid

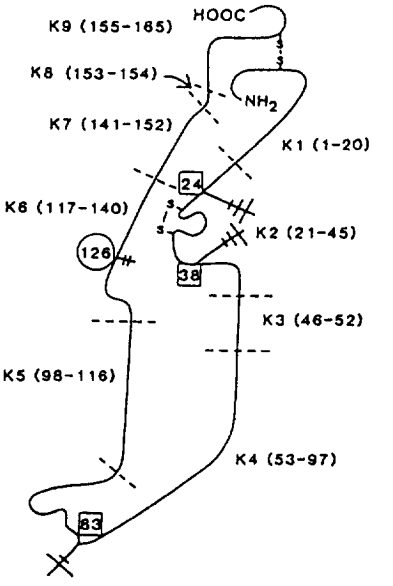

Fig. 2. Predicted structure of human erythropoietin. K1–K9 are peptides generated by endoLys-C, and the amino acid residue numbers are indicated in parentheses. The *N*-linked saccharides are located at the amino acid residues 24, 38 and 83, whereas *O*-linked saccharide is located at the residue 126 (reprinted with permission from Ref. [83]).

Table 1
Characteristics of blood

	Anemia	Normal	Polycythemia
Hematocrit	<40%	40–45%	60–70%
Hemoglobin	<14 g/dl	14–16 g/dl	<16 g/dl
Number of RBC	$<5\cdot10^6$/mm^3	$5\cdot10^6$/mm^3	$6–8\cdot10^6$/mm^3
Reticulocyte	<1%	1.16%	<1.2%
Erythropoietin	<130 pg/ml	130–230 pg/ml	<230 pg/ml

residues. The *N*-linked carbohydrates of EPO have sialic acids at the terminal part, whereas the *O*-linked (mucin type) carbohydrate of EPO does not have the sialic acids [28,30,41]. Thus, the *N*-linked carbohydrates are necessary for in vivo activity but are not necessary for in vitro activity. The *N*-linked carbohydrates are incorporated for proper biosynthesis and/or secretion, for stability and for biologically active functioning. Higher branching of the *N*-linked carbohydrate with terminal sialic acid is essential for the effective expression of in vivo biological activity of EPO [31,32].

2.3. Recombinant human EPO (rHuEPO)

A major problem in exogenous EPO analysis is the existence of a minute, but significant, quantity of natural EPO in the body. To obtain EPO in quantity, human cDNA clones have been isolated in several laboratories [9–11,42]. The expression of EPO cDNA clones has also been achieved by transfecting mammalian cells (either Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cell lines [43]. Since no native host for EPO is available, the above process is the only way to produce rHuEPO in quantity. Thus, rHuEPO is classified as the third generation of biomedicine [44]. The produced rHuEPO is homogenous with respect to the peptide sequence of natural EPO, but rHuEPO contains heterogeneous carbohydrate moiety [41,45,46]. In spite of the difference in carbohydrate structure when compared to natural EPO, rHuEPO has been successfully used to treat anemia (as an end-stage disease) resulting from AZT therapy used for HIV infection in humans [18]. Because of possible EPO misuse by athletes (due to the physiological effects of EPO) [2,4,6], a specific and sensitive analytical biotechnique is required to identify the administration of rHuEPO.

3. Characterization

For therapeutic utility of proteins as a biomedicine, macromolecular structure of the interesting protein has to be characterized for the intact protein, for proteolytic digests, for sequence information and for post-translational modifications [47–51]. A general strategy for the characterization of the interesting protein is as follows. Firstly, the target protein, either natural or recombinant, should be purified. The obtained pure and intact protein is characterized by: high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), electrospray ionization mass spectrometry (ESI-MS), matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) and/or nuclear magnetic resonance (NMR) to determine molecular mass and homogeneity [46,47,50,52–54]. Moreover, the target protein can be identified and resolved by its relative molecular mass [32,55–58] through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. Secondly, after digesting the intact protein with protease, the complexes of enzymatic digests are separated to identify fractionated glycopeptides. The fractionated glycopeptides are characterized by HPLC, HPCE, MALDI-TOF-MS ^{1}H NMR and/or ESI-MS [48,49,59–62]. The individual glycopeptides are then deglycosylated to form peptides and oligosaccharides, respectively [63,64]. Amino acid sequences can be obtained from the resulting peptides [65], while carbohydrate moieties can be obtained by analyzing the resulting oligosaccharides [66–71]. Finally, by combining all the acquired information, the molecular structure of the target protein, EPO, is deduced.

3.1. Purification

Natural EPO was purified from biological matrices, such as urine and serum, to obtain EPO in mass for further study. Since EPO is present at picomolar levels in biological samples, purification is difficult and its yield is very low (about 20%) [24]. Either by successive chromatography or by immunoaffinity chromatography, natural EPO was purified from urine [24,26]. About 1 mg of pure natural EPO was obtained from 2550 l of urine after seven successive steps: anion-exchange chromatography (DEAE-cellulose column), phenol *p*-salicylate treatment, ethanol precipitation (90% cut-off), anion-exchange chromatography (DEAE–agarose column), adsorption chromatography (sulfopropyl–Sephadex column), gel permeation chromatography (Sephadex G-100 column) and hydroxylapatite chromatography

[24]. Alternatively, about 6 mg of natural EPO was isolated from 700 l of urine using immunoaffinity chromatography (monoclonal antibodies against EPO fixed on Affi-Gel 10) and gel filtration chromatography (Sephadex G-100 column) [26]. Thus, natural EPO was purified efficiently by immunoaffinity chromatography.

Recombinant human EPO was purified from the transfected cell lines using a combination of procedures previously described for the purification of natural EPO [25,31,33]. By expressing a cDNA clone of the human gene, 1 l of the conditioned medium of a CHO cell line normally yields approximately 30–80 mg of rHuEPO [10]

3.2. Biological analysis

EPO concentration in body fluids was determined by biological analyses such as exhypoxic polycythemic mouse assay, enzyme linked immuno selective assay (ELISA) and radioimmunoassay (RIA) [72–77]. Biological analysis is sensitive, accurate, precise, specific and reproducible; unfortunately, the analysis is difficult, long, expensive and even needs radioisotope. Recently, reticulocyte measurement that does not require radioisotope has been developed for analysis of EPO [78]. The specifications of each method are shown in Table 2.

3.2.1. Exhypoxic polycythemic mouse assay

Measuring EPO concentration in serum is useful, not for evaluating various anemic conditions but for monitoring EPO administration in vivo. The exhypoxic polycythemic mouse assay is most widely used for the analysis of EPO in vivo. By hypobaric exposure of mice at 0.42 atm for 2 weeks, the induced polycythemia results in the incorporation of radioactive iron (^{59}Fe) into newly formed red blood cells. EPO administration can be evaluated by measuring radioactive EPO with a gamma counter [75,78]. Thus, the polycythemic assay must use radioisotopes and takes 3 weeks to obtain results.

Table 2
The specification of bioassay for erythropoietin

Bioassay	Time	LOD of EPO	References
RIA	2 days	0.5 U/l	[73]
	2 days	3.0 U/l	[75]
Reticulocyte count	1 week	4.0 U/l	[78]
Polycythemic assay	3–4 weeks	0.1 U/mouse	[75]

3.2.2. Radioimmunoassay (RIA)

A RIA of EPO in serum has been described in which highly purified rHuEPO was used as the immunogen and standard [75,79]. Also, there is a commercial RIA kit available for determining EPO in serum or plasma [73]. RIA is based on the competition between EPO in serum and [^{125}I]rHuEPO for an antibody raised from purified human urinary EPO [80]. The second IRP (International Reference Preparation, WHO) is usually used as the standard [81]. The lower limit of detection (LOD) of RIA is 3 mU EPO/ml of serum.

3.2.3. Reticulocyte counts

Since the increasing number of reticulocytes in peripheral circulation has been indicated as an early marker for EPO activity and as the remaining nuclear reticulum makes the reticulocyte easily recognizable by microscopy, the percentage of reticulocyte in peripheral blood has been recently determined by thiazole orange staining and flow cytometric count analysis [78]. The reticulocyte increase is evident with two or three doses subcutaneously (one dose is 20 units/mouse) of rHuEPO in vivo and a significant increase in reticulocyte counts can be achieved by days 3 or 4, so that a complete assay can be run in a single week. Although less precise than exhypoxic polycythemic mouse assay for EPO determination in serum, reticulocyte counts are rapid, easy, and reproducible enough to conduct batch testing of commercially prepared recombinant EPO products. Thus, reticulocyte counts can be used to monitor the therapeutic effect of EPO as well as to determine EPO concentration in serum.

3.3. Structural analysis

There is similar homology between natural EPO and rHuEPO except carbohydrate moiety. Structural analysis of the heterogeneous carbohydrate moiety attached to EPO is essential for distinguishing rHuEPO from natural EPO as well as for investigating the functional role of EPO. As mentioned in Section 3, after several successive steps, the obtained

oligosaccharide from EPO is analyzed by ESI-MS, FAB-MS, and ^{1}H NMR. The characteristic structures of EPO can be elucidated by combining all the collected information.

3.3.1. Peptide mapping

To address EPO structure, the very first thing to do is peptide mapping. Usually, peptide mapping of EPO was done by proteolytic enzymes such as endoLys-C, endoGlu-C and trypsin. After enzymatic digestion, reversed-phase HPLC (RPC) or HPCE is needed to separate and to fractionate the enzyme digests [58,59]. The number of fractions caused by differences in digestion and chromatography is dependent upon enzyme characteristics and the three dimensional structure of the native protein. Typically, by RPC fractionation, 8–9 enzymatic peptide peaks are obtained and are classified into two groups, non-glycosylated peptide and glycosylated peptide. Non-glycosylated peptide has a sharp peak, while glycosylated peptide has a broad peak and usually elutes later than non-glycosylated peptide due to polar carbohydrate moiety. As shown in Fig. 3, the broad peaks such as peak 3, peak 5 and peak 9 are glycopeptides that have been digested with endoLys-C followed by RPC. The peaks were also confirmed by determining the sequence of all the peptides [21,62]. The *N*-linked glycopeptides are located at peak 3 (Glu21 to Lys45) and peak 9. The *O*-linked glycopeptide is located at peak 5 (Glu117 to Arg140).

3.3.2. Carbohydrate microheterogeneity

Carbohydrate microheterogeneity of EPO is due to post-translational modification, especially glycosylation [30,50]. Since glycosylation occurs differently depending on the host, glycosylation may be greatly influenced by the tertiary protein structure and the carbohydrate chain at the neighboring site [51,82]. Thus, the carbohydrate structure of rHuEPO may be different from that of natural EPO, as rHuEPO is produced from heterogeneous mammalian cell lines [9,10]. This natural heterogeneity of glycosylation in EPO portrays biological properties of EPO; therefore, carbohydrate microheterogeneity is possibly a marker for identifying rHuEPO administration. Two major approaches have been employed for addressing carbohydrate microheterogeneity; one approach is peptide mapping followed by deglycosylation to obtain oligosaccharide from each glycosylation site [62] and the other approach is deglycosylation of intact EPO to obtain oligosaccharides [25,28,31,45,46,64].

As shown in Fig. 4, *N*-linked carbohydrate units (such as biantennary, triantennary, and tetraantennary moieties) present in EPO are very diverse. The ratio of the di-, tri- and tetra-antennary moieties is 1.4:13.5:85.1, respectively [25,83]. Thus, among such large and branched structures, the main components of the *N*-linked carbohydrates of EPO are tetraantennary saccharides with or without *N*-acetyllactosaminyl repeats [25,45,46]. Sialic acid (*N*-acetylneuraminic acid: NeuAc) is α2-3 linked to all the saccharides since α2-6 substituted galactose derivative was not detected and activity of sialyltransferase correlates with α2-3 linkage of NeuAc. The degree of sialylation is about 80–97% [44,63,64]. The structure of the asialo tetraantennary saccharide and triantennary saccharide core with one *N*-acetyllactosaminyl repeat is composed of

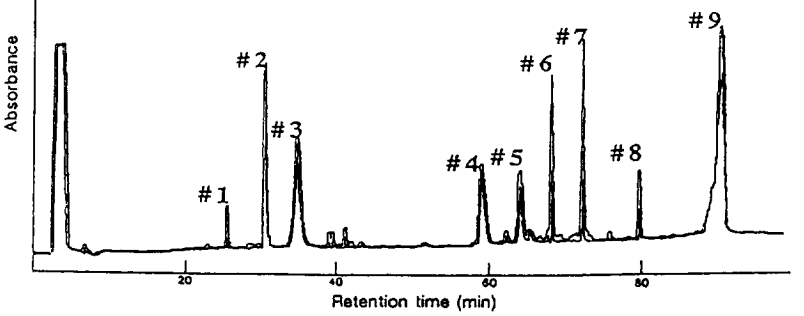

Fig. 3. Peptide mapping of human erythropoietin. Nine peptides are resolved by reversed-phase HPLC of the digests of EPO by endoLys-C. Peaks #3, #5 and #9 are identified as glycopeptides (reprinted with permission from Ref. [62]).

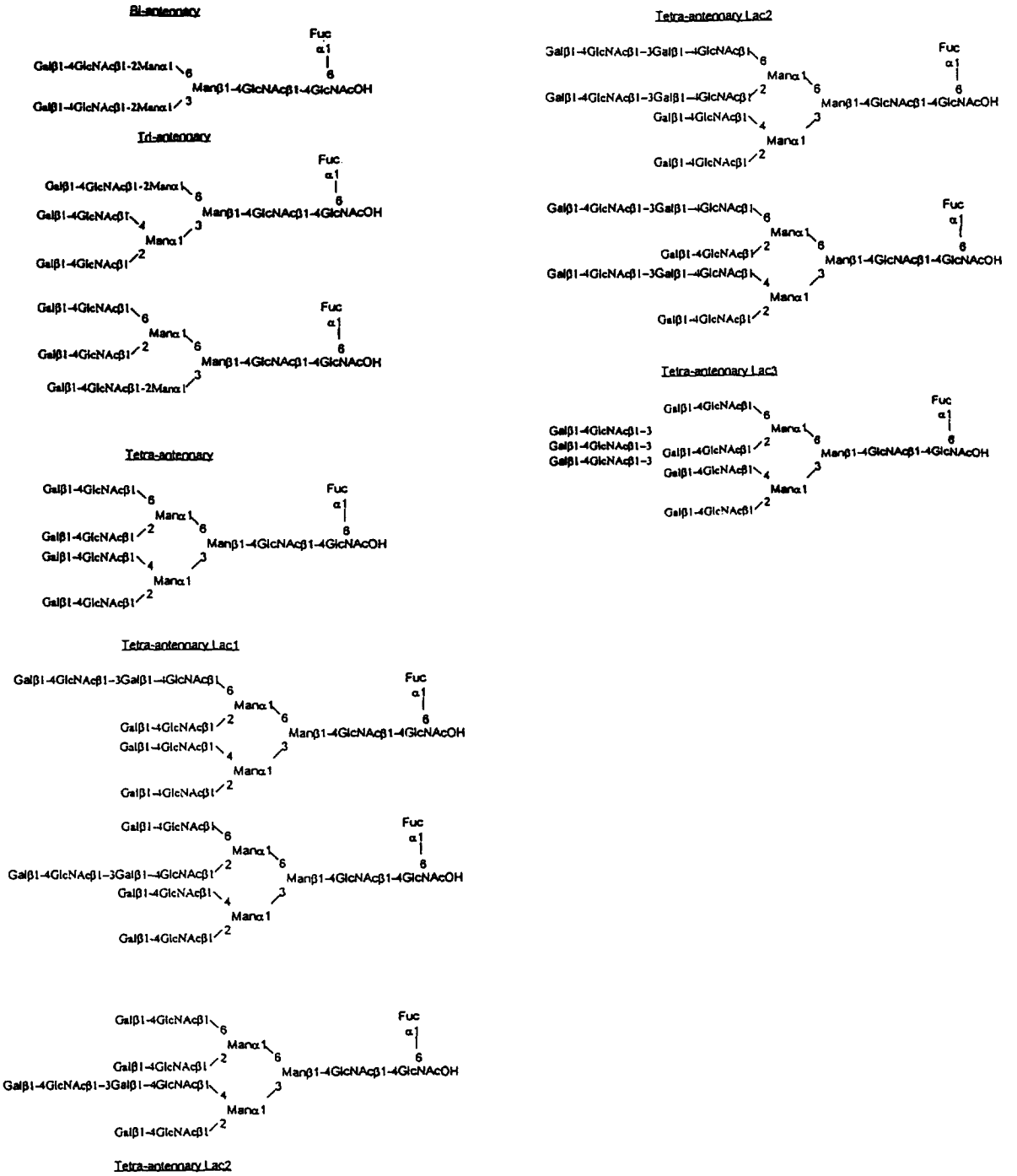

Fig. 4. Characteristic structure of *N*-linked oligosaccharides of erythropoietin (reprinted with permission from Ref. [83]).

Man1-6(Man1 - 3)Manβ1 - 4GlcNAcβ1 - 4(±Fucα1-6)GlcNAcOH that has been confirmed by methylation analysis and FAB-MS [25]. When *N*-linked carbohydrates were permethylated and analyzed by ES-MS, the profiles of the ions represent complexity by showing a distribution of bi-, tri-, and tetra-antennary moieties with variations in the number of lactosaminyl residue and sialic acid capping [62]. As a result, the most abundant ion, *m/z* 1544.5 is a tetraantennary oligosaccharide in the three charged state that is located at Asn83, displayed in Fig. 5A. In addition, depending on the number of *N*-acetyllactosaminyl repeats, homologues are shown at *m/z* 1694.4 (one), and *m/z* 1844.4 (two). As depicted in Fig. 5B and 5C, *m/z* 1574.3, and *m/z* 1724.6 represent the trineuraminyl capped series for *N*-linked oligosaccharide connected at Asn38 and Asn 24.

The *N*-linked carbohydrate moiety on EPO has been classified into three functional units: (i) the core part (Man3GlcNAc(±Fuc)GlcNAc) that maintains the bioactive conformation of the polypeptide, (ii) the branching part (GlcNAcs) that encodes a signal to the target organ and (iii) the terminal part (sialic acids, Gal, GalNAc, *N*-acetyllactosamine repeats and/or Galα1-3Gal, etc) that determines the lifetime of glycoproteins in the blood stream through lectin-mediated clearance systems [44]. Therefore, the oligosaccharide portion of EPO, although required for action in vivo, is not required for interaction with the target cells of the blood forming system.

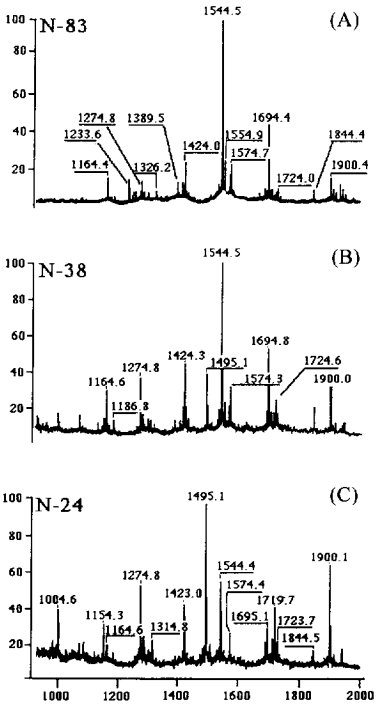

Fig. 5. ES-MS of methylated *N*-linked saccharides following glycopeptide deglycosylation. N-83 (A), N-38 (B) and N-24 (C), respectively (reprinted with permission from Ref. [62]).

Recently, *O*-linked oligosaccharide has also been characterized by using a combination of bioanalytical techniques previously described for the characterization of *N*-linked oligosaccharides. The main *O*-linked oligosaccharide at Ser126 is a branched triantennary saccharide that contains the Galβ1-3GalNAc core structure with one NeuAcα2-3 linked to Gal or a second NeuAc attached α2-6 to GalNAc [62,63]. The *O*-linked carbohydrate moiety may not be required in the biological activity of the hormone EPO in vivo or in vitro, respectively.

3.3.3. Comparative analysis of natural EPO and rHuEPO

Human urinary EPO (uEPO) migrates as a diffuse band at 34 000–38 500 Da, and rHuEPO migrates at 32 000–38 000 Da by sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) [25]. On SDS-PAGE, uEPO and rHuEPO migrate slightly differently. However, they are almost homologous in peptide sequence. Natural EPO and rHuEPO both consist of one polypeptide chain of 165 amino acids. The polypeptide is proteolytically processed at C-termini that are each missing the COOH-terminal Arg166 amino acid residue [9,15]. Both uEPO and rHuEPO also contain heterogeneous carbohydrates, although with slightly different sialylation as shown in Table 3 [25,41,45]. By comparing the characteristic structure of *N*-linked saccharides obtained from the bioanalytical method, a few differences have been elucidated [15,45,46]. Firstly, both uEPO and rHuEPO have a significant amount of tetraantennary oligosaccharide with Gal(β1-3)GlcNAc. Secondly, uEPO apparently lacks biantennary saccharides, but rHuEPO contains heterogeneous biantennary sac-

Table 3
Carbohydrate composition of erythropoietin

	Carbohydrate composition (mol/mol)				
	N-Acetylhexosamine	Mannose	Fucose	Galactose	Sialic acid
uEPO	22.1	8.1	2.6	13.3	10.7
rHuEPO	28.9	10.1	3.3	15.7	18.7

charides depending upon the batches. Thirdly, uEPO lacks the tetraantennary structure with *N*-acetyllactosaminyl repeats that can be found in rHuEPO. Finally, unlike rHuEPO, natural uEPO contains no detectable amount of triantennary saccharides with Gal(β1-4)GlcNAc.

4. Conclusion

Using the hypothesis that both natural EPO and rHuEPO contain heterogeneous carbohydrates with slightly different sialylation, the structural characterization of EPO has been differentiated based on carbohydrate structure. The results suggest that natural EPO contains heterogeneous carbohydrate saccharides different from rHuEPO. The results likely indicate that characteristic carbohydrate structure may be an important factor for identifying rHuEPO administration in the blood stream.

5. List of abbreviations

BKH	Baby hamster kidney
CHO	Chinese hamster ovary
ELISA	Enzyme-linked immuno-selective assay
EPO	Erythropoietin
rHuEPO	Recombinant human erythropoietin
uEPO	Urinary erythropoietin
ESI	Electrospray ionization
Hb	Hemoglobin
Hct	Hematocrit
HIV	Human-induced virus
HPLC	High-performance liquid chromatography
HPCE	High-performance capillary electrophoresis
IOC	International Olympic Committee
LOD	Low detection limit
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometer
PAGE	Polyacrylamide gel electrophoresis
RBC	Red blood cell
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulfate
Tfr	Transferrin soluble receptor
TOF	Time of flight

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