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Carbohydrate Structure of N- and O-linked Oligosaccharides of Human Erythropoietin Expressed in Chinese Hamster Ovary Cells

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Abstract: A recombinant human erythropoietin (EPO), expressed in Chinese hamster ovary (CHO) cells, is glycosylated at Asn 24, Asn 38, Asn 83, and Ser 126. After release of the N-linked carbohydrate chains by peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase F, the oligosaccharides were analyzed by FACE (Fluorophore-Assisted Carbohydrate Electrophoresis). The O-linked carbohydrate chain was separated by hydrazine, and analyzed by FACE. The monosacccharide composition of recombinant EPO showed mannose, fucose, galactose, N-acetylglucosamine, N-acetylneuraminic acid, and a trace of N-acetylgalactosamine, which are typical monosaccharides in the glycoproteins from the CHO cell. Sequences of N-linked and O-linked oligosaccharides were determined. The structure and composition of oligosaccharides attached to recombinant human EPO, expressed in the CHO cell, are identical to the reported oligosaccharide structure in human EPO isolated from urine.

Key words: carbohydrate structure, erythropoietin.

Erythropoietin (EPO) is a glycoprotein, which binds to its receptor on erythroid progenitor cells, thereby stimulating cell differentiation and proliferation. Deglycosylated EPO shows *in vitro* activity, but decreased *in vivo* activity, which implicates the importance of carbohydrates on EPO activity (Dordal *et al.*, 1985).

Human EPO contains three N-linked carbohydrate chains and one O-linked oligosaccharide, which are located on Asn 24, Asn 38, Asn 83, and Ser 126.

The importance of carbohydrate structure in glycoprotein was well visualized in the studies of glycoprotein hormones. It was reported that the removal of the carbohydrates from subunit of hCG, human chorionic gonadotropin, confers some conformational change (Strickland and Puett, 1982). In addition, free α subunit expressed in mammalian cells has a triantennary structure of carbohydrates, while α subunit in dimer isolated from urine possesses the biantennary oligosaccharide (Lustbader *et al.*, 1987). The deglycosylated hCG binds strongly to cell surface receptors, but lacks the ability to induce biological responses such as activation of adenylate cyclase and steroidogenesis (Moyle *et al.*, 1975).

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Urine EPO was known to have α and β forms, which were recognized by hydroxylapatite column chromatoggraphy (Miyake *et al.*, 1977). Later, the structure of both forms was reported, in which the composition of monosaccharides was the same, but the difference was in their quantities (Dordal *et al.*, 1985).

In the present study, the structure of carbohydrate chains from recombinant human EPO has been studied by the FACE method.

Experimental Procedures

Materials

The monosaccharide compositional analysis starter kit, N-linked oligosaccharide profiling starter kit, O-linked oligosaccharide profiling starter kit, N-linked oligosaccharide sequencing kit, and O-linked oligosaccharide sequencing kit were obtained from Glyko Inc. (Novato, USA). PNGase F (a recombinant enzyme from *Flavobacterium meningosepticum* and expressed in *E. coli* by Glyko), sialidase (a recombinant enzyme and expressed in *E. coli*, α2-3, 6, 8 specific (EC 3.2.1.18)) and O-glycosidase(endo-α-N-acetylgalactosaminidase (EC 3.2.1.97) from *Diplococcus pneumoniae*) were purchased from Boehringer Mannheim (FRG). Acrylamide, bis-acrylamide, glycine, and Tris-HCl were bought from Sigma (St.

Louis, USA).

Expression of EPO

Genomic DNA of EPO was transfected into CHO cells. After selection of clones for growth in increasing concentrations of methotrexate, EPO expression was observed by ELISA using monoclonal antibody and the thymidine uptake assay method. CHO cells which express EPO continuously were maintained in a culture flask, and part of the cells were frozen and kept in a N_2 tank.

Purification of EPO

EPO was purified from the culture media of CHO cells by sequential chromatography using the procedures which were reported previously (Miyake *et al.*, 1977). Purified EPO showed a single band on the acrylamide gel stained by the silver nitrate method (Bassam *et al.*, 1991).

Release of the N-linked carbohydrate chains

Purified EPO (650 μg) was lyophilized in a microfuge tube and sodium phosphate buffer (25 μl, 100 mM, pH 7.5) was added. PNGase F (10 mU) was added to the reaction mixture and incubated for 2 h at 37℃.

Release of the O-linked carbohydrate chains

PNGase F-treated EPO (650 μg) was lyophilized in an airtight screw cap tube. 0.8 ml of anhydrous hydrazine was added using a glass pipet. Using a dry heat block, the sample was boiled for 10 h at 100℃. Hydrazine was removed by evaporation.

Fluorophore labelling

Isolated oligosaccharides were labelled by reductive amidation with ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) (Jackson and Williams, 1991).

Electrophoresis

Polyacrylamide gel electrophoresis was performed without sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (Schaumann *et al.*, 1993). The lower gel was composed of 40% acrylamide and 5% bis-acrylamide while the upper gel was made of 5% acrylamide and 5% bis-acrylamide. Gels were run for 3~5 h at 4℃ at 350 V.

Monosaccharide analysis

EPO (10.26 μg) was divided into three 3.42 μg aliquots for the separate determinations of neutral and amino sugars, and sialic acids, respectively. After lyophilization, neutral monosaccharides were hydrolyzed by dissolving EPO (3.42 μg) in 100 μl of 2 M TFA at 100℃ for 5 h. N-acetylneuraminic acids were hydrolyzed by dissolving 3.42 μg in 100 μl of 0.1 M TFA at 80℃ for 1 h. Amino sugars were hydrolyzed by dissolving 3.42 μg in 100 μl of 4 M HCl at 100℃ for 3 h (Starr *et al.*, 1996).

For quantitation, monosaccharides standards were also applied: GalNAc 81 pmol, Mannose 59 pmol, Fucose 61 pmol, Glucose 67 pmol, Galactose 60 pmol, and GlcNAc 72 pmol per one lane. After electrophoresis, image analysis was performed using a densitometer (Glyko Inc.).

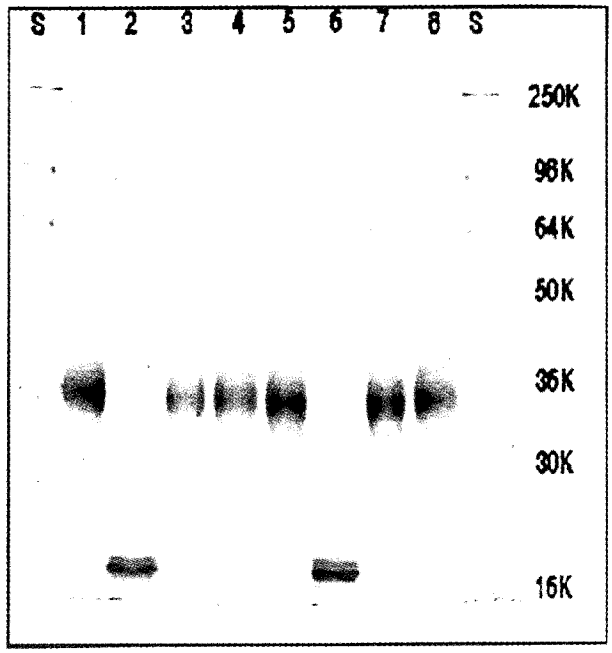

Fig. 1. Deglycosylation of human recombinant EPO. 15 μg of EPO (lane 1 & 8) was treated with neuraminidase (lane 7), PNGase F (lane 2), Endoglycosidase H (lane 3), O-glycosidase (lane 4), PNGase F and O-glycosidase (lane 6), and sialidase and O-glycosidase (lane 5). After deglycosylation, the mobility of the bands were changed. Lanes s: molecular weight standard (from the top; Rabbit skeletal muscle myosin, *E. coli* β-galactosidase, Rabbit muscle phosphorylase B, Bovine serum albumin, Hen egg white ovalbumin, Bovine carbonic anhydrase, Soybean trypsin inhibitor, Hen egg white lysozyme, Bovine pancreas aprotinin).

Sequencing of the N-linked oligosaccharide

From the N-linked oligosaccharide profile (Fig. 2), four bands were isolated and the oligosaccharides were separated, respectively. Isolated oligosaccharides were fluorophore-labelled and treated with neuraminidase, β-galactosidase (EC 3.2.1.23) from *E. coli*, hexosaminidase and α-mannosidase (α1-2, 3, 6 specific), sequentially. The modified oligosaccharides were electrophoresed.

Sequencing of the O-linked oligosaccharide

Three bands were isolated from the O-linked oligo-

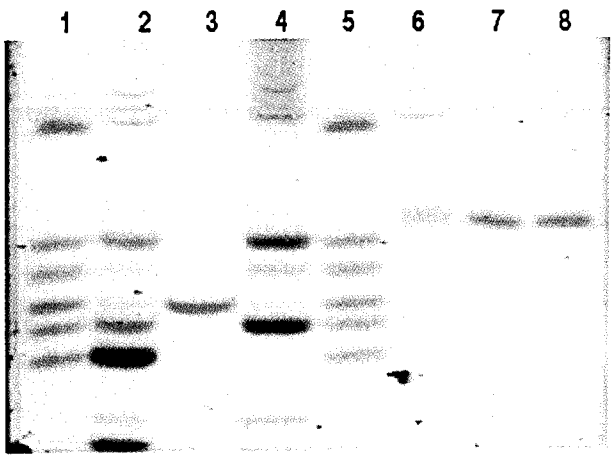

Fig. 2. Monosaccharide analysis of human recombinant EPO. 3.42 μg of EPO was analyzed after amine hydrolysis, neutral hydrolysis, or sialic acid hydrolysis. Lanes 1, 5 : monosaccharide ladder standard (from top; N-acetylgalactosamine, mannose, fucose, glucose, galactose, and N-acetylglucosamine), 2: after amine hydrolysis, 3: glucose standard, 4: after neutral hydrolysis, 6: after sialic acid hydrolysis, 7, 8: NANA standard.

Table 1. Monosaccharide analysis of human recombinant EPO. 3.42 μg of EPO was analyzed after amine hydrolysis, neutral hydrolysis, or sialic acid hydrolysis

Monosaccharides	pmol/lane	Molar ratio (%)
GalNAc	0	0
Mannose	111.96	16.8
Fucose	38.24	5.7
Glucose	0	N/A[a]
Galactose	169.49	25.4
GlcNAc	239.95	36.0
NANA	107.27	16.1

[a] N/A : not applicable.

saccharide profile (Fig. 3). After extraction of the oligosaccharides from the gel, neuraminidase were treated. The enzyme treated oligosaccharides were electrophoresed.

Results and Discussion

Carbohydrates from a glycoprotein show so much heterogeneity even secreted from a cloned cell. The reason is not obvious, although two speculations may be possible. One is a lack of fidelity of the post-translational modification process and the other is a fine tuning mechanism which supplies the heterogeneity that may be necessary for complete *in vivo* activity (Geisow *et al.*, 1992).

The broad band of EPO in SDS-PAGE may be derived from its difference in carbohydrate structure. Removal of carbohydrate chains resulted in narrow and sharp

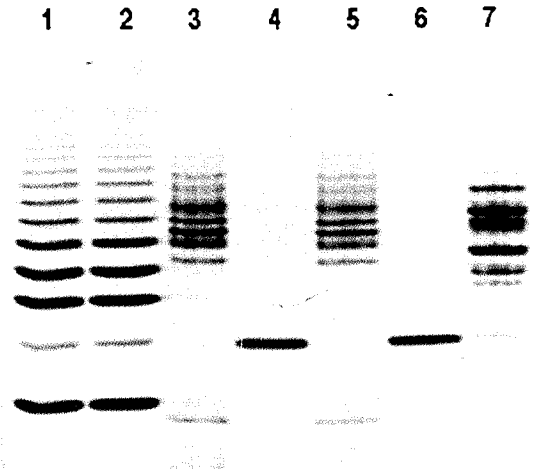

Fig. 3. N-linked profile of human recombinant EPO. EPO was treated by the method described in the experimental procedures. Lanes 1, 2: wheat starch polymers, 3, 5: N-linked oligosaccharides separated from human recombinant EPO, 4, 6: maltotetraose, 7: control glycoprotein (chicken egg trypsin inhibitor).

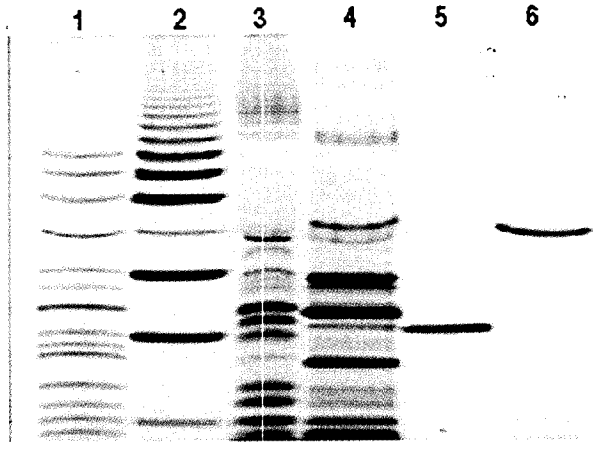

Fig. 4. O-linked profile of human recombinant EPO. EPO was treated by the method described in the experimental procedures. Lanes 1: standard monosaccharides and oligosaccharides (from top; maltoheptaose, maltohexaose, maltopentaose, maltotetraose, maltotriose, cellotriose, galactobiose, maltose, lactose, galactosylgalactose, N-acetylgalactosamine, galactose, glucose, and 6-deoxyglycose), 2: wheat starch polymers, 3: O-linked oligosaccharides separated from human recombinant EPO, 4: control glycoprotein (chicken egg trypsin inhibitor), 5: Gal-GalNAc standard, 6: maltotetraose.

bands (Fig. 1). Band shift was also observed, which implicates most of the EPO molecules are N-glycosylated. Endoglycosidase H failed to cleave the carbohydrate from rhEPO, hence, it is possible to speculate that no oligomannose type or hybrid type of oligosaccharides are in rhEPO.

From 3.42 μg of rhEPO, a total of 1.313 μg of monosaccharides were detected. Therefore it could be calculated that the carbohydrate content of rhEPO is 38.4 % (w/w). The carbohydrates are composed of mannose,

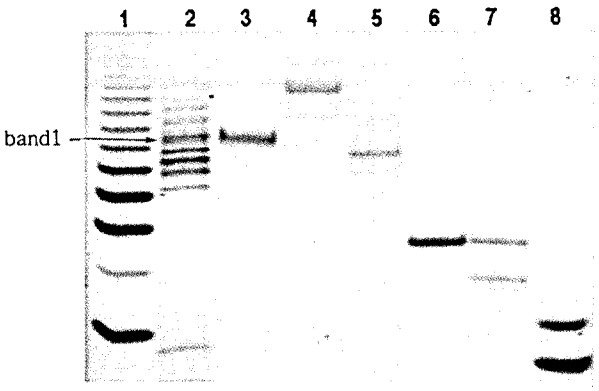

Fig. 5. Sequencing gel of isolated band No. 1 from N-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: N-linked profile of human recombinant EPO, 3: isolated band 1, 4: band 1+neuraminidase, 5: band 1+neuraminidase+β-galactosidase, 6: band 1+neuraminidase+β-galactosidase+hexosaminidase, 7: band 1+neuraminidase+β-galactosidase+hexosaminidase+α-mannosidase, 8: N-linked core standard.

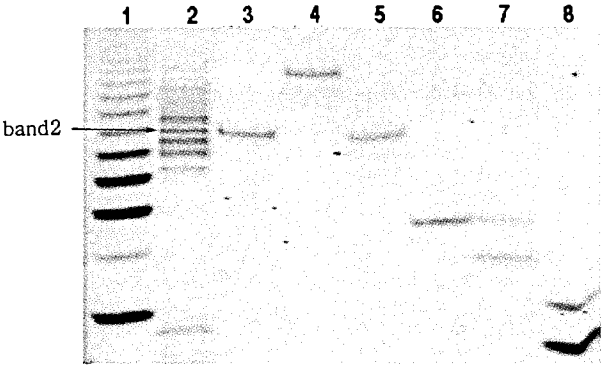

Fig. 6. Sequencing gel of isolated band No. 2 from N-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: N-linked profile of human recombinant EPO, 3: isolated band 2, 4: band 2+neuraminidase, 5: band 2+neuraminidase+β-galactosidase, 6: band 2+neuraminidase+β-galactosidase+hexosaminidase, 7: band 2+neuraminidase+β-galactosidase+hexosaminidase+α-mannosidase, 8: N-linked core standard.

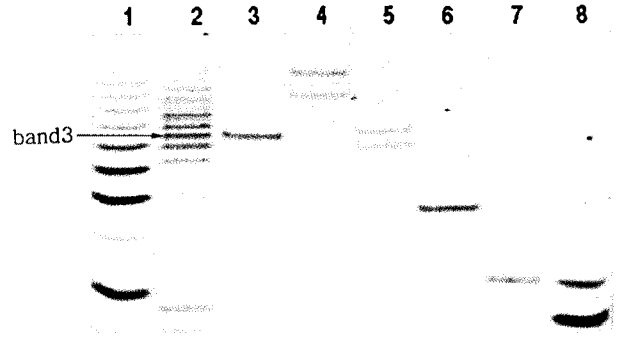

Fig. 7. Sequencing gel of isolated band No. 3 from N-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: N-linked profile of human recombinant EPO, 3: isolated band 3, 4: band 3+neuraminidase, 5: band 3+neuraminidase+β-galactosidase, 6: band 3+neuraminidase+β-galactosidase+hexosaminidase, 7: band 3+neuraminidase+β-galactosidase+hexosaminidase+α-mannosidase, 8: N-linked core standard.

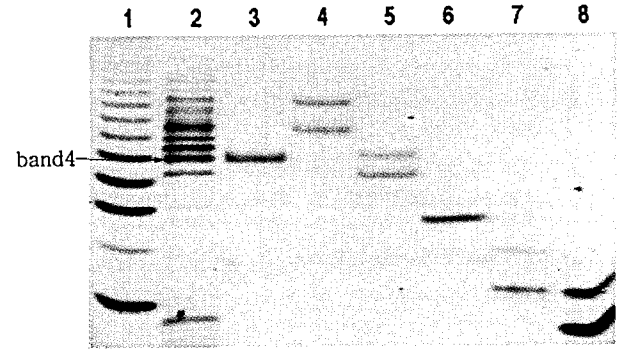

Fig. 8. Sequencing gel of isolated band No. 4 from N-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: N-linked profile of human recombinant EPO, 3: isolated band 4, 4: band 4+neuraminidase, 5: band 4+neuraminidase+β-galactosidase, 6: band 4+neuraminidase+β-galactosidase+hexosaminidase, 7: band 4+neuraminidase+β-galactosidase+hexosaminidase+α-mannosidase, 8: N-linked core standard.

fucose, galactose, N-acetylglucosamine, N-acetylneuraminic acid and a trace of N-acetylgalactosamine (Table 1). Even though N-acetylgalactosamine was not detected in this monosaccharide analysis, it was found after a sequencing reaction of O-linked carbohydrate (Fig. 9 & 10). Maybe the usage of a small amount of EPO resulted in a failure of detection. The same results were also reported during the analysis of the carbohydrate from human urinary EPO (Dordal *et al.*, 1985). From these results, the rhEPO, expressed in CHO cells, seems to be an α-form (Table 1).

After image analysis of the gel, the monosaccharide content was detected. The molar ratio of each monosaccharide is fucose : mannose : galactose : NAcGlc : NANA =382 pmol : 1120 pmol : 1695 pmol : 2400 pmol : 1073 pmol (=1 : 2.95 : 4.46 : 6.32 : 2.82). That implicates the approximate structure of the oligosaccharides in EPO; major type carbohydrate contains 1 fucose, 3 mannoses, 4~5 galactoses, 6 NAcGlcs, and 3 NANAs. And this speculation became true after the sequencing of N-linked oligosaccharide chains. The major part of the oligosaccharides from EPO has a tetraantennary carbohydrate structure. A molecule of EPO contains 3~4 fucose molecules that implicates the possibility of fucosylation of all oligosaccharides in the molecule.

Structure determination of the N-linked oligosaccharides of EPO

After an analysis of four bands of N-linked oligosaccharides, we found that band 1 is a disialylated, galactosylated, tetraantennary, core-fucosylated oligosaccharide (Fig. 5) and band 2 is a trisialylated, galactosylated, tetraantennary, core-fucosylated oligosaccharide (Fig. 6). Band 3 is a mixture of tetrasialylated, galactosylated,

Table 2. Composition of N-linked oligosaccharides of human recombinant EPO. The type of N-linked oligosaccharides were deduced from the results of the carbohydrate sequencing

Band No.	Content (%)	Structure
1	31.9	$(NANA)_2$-$(Gal$-$GlcNAc)_n$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc
2	18.4	$(NANA)_3$-$(Gal$-$GlcNAc)_4$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc
3-1[a]	12.2	$(NANA)_4$-$(Gal$-$GlcNAc)_4$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc
3-2	9.7	$(NANA)_2$-$(Gal$-$GlcNAc)_3$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc
4-1[b]	6.5	$(NANA)_3$-$(Gal$-$GlcNAc)_3$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc
4-2	8.9	$(NANA)_1$-$(Gal$-$GlcNAc)_2$-$(Man)_3$-GlcNAc-[Fuc]-GlcNAc

n=5~7

[a,b] Since bands 3 and 4 are the mixture of two bands (Figs. 7 & 8), each band is noted as 3-1, 3-2, 4-1 and 4-2.

tetraantennary, core-fucosylated oligosaccharide and a disialylated, galactosylated, triantennary, core-fucosylated one (Fig. 7). Band 4 contains a mixture of trisialylated, galactosylated, triantennary, core-fucosylated oligosaccharide and monosialylated, galactosylated, biantennary, core-fucosylated carbohydrate (Fig. 8). As expected in the monosaccharide analysis, most of the core oligosaccharides were fucosylated.

Band 1 in the N-linked oligosaccharide profile is probably a mixture of tetraantennary oligosaccharides, some of which have N-acetyllactosamine repeating units (Fig. 3). After neuraminidase treatment (lane 4), band 1 became one major band and three minor bands (Fig. 5). This multi-band pattern leads us to believe that the sialidase reaction might have been incomplete and that some of the tetraantennary branches have additional N-acetyllactosamine repeating units. The presence of this repeating unit was reported in the tetraantennary structure, and it is not correlated with the *in vivo* activity of EPO (Takeuchi *et al.*, 1989). Lane 5 of Fig. 3 also posseses 4 bands, which means that, though β-galactosidase was treated, minor bands were not removed. It supports strongly the presence of extra lactosamine repeating units in band 1. It seems that the mannosidase reaction was not complete in Fig. 7 and 8 (lane 7), but, was complete in Fig. 9 and 10 (lane 7).

Two different types of N-linked oligosaccharides were found in tPA: a "complex type" and "oligomannose type". The former one has sialic acid in its termini and the latter contains terminal mannose. Glycosylation patterns of rhEPO have been analyzed by ^{1}H-NMR (Hokke *et al.*, 1995) and carbohydrates both from human urine and CHO cell-derived EPO were also analyzed by HPLC and fast atom bombardment mass spectrometry (Sasaki *et al.*, 1987). In agreement with these reports, results from the FACE analysis showed that

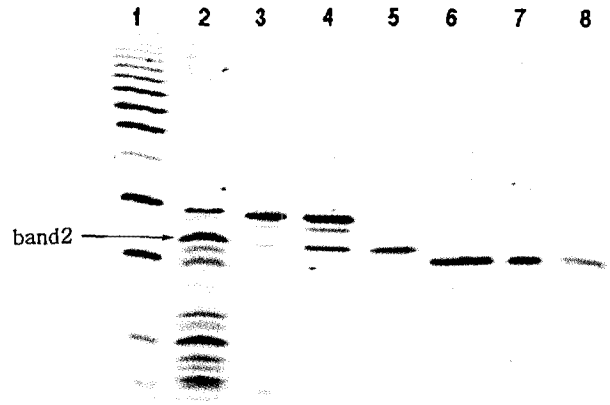

Fig. 9. Sequencing gel of isolated bands No. 2 and No. 3 from O-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: O-linked profile of human recombinant EPO, 3: di-sialylated Gal-GalNAc standard, 4: partial neuraminidase digest of di-sialylated Gal-GalNAc standard, 5: isolated band 2, 6: band 2+neuraminidase, 7: Gal-GalNAc standard, 8: isolated band 3.

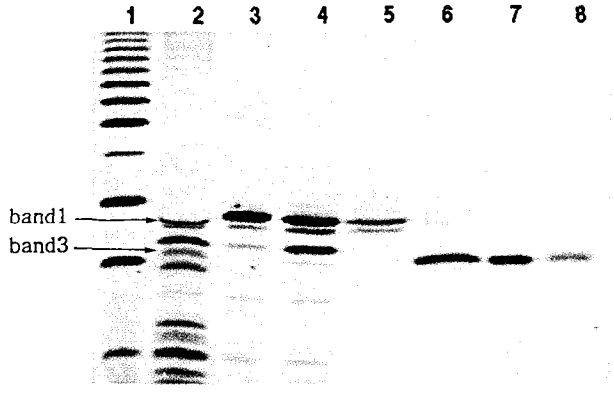

Fig. 10. Sequencing gel of isolated bands No. 1 and No. 3 from O-linked profile of human recombinant EPO. Lanes 1: wheat starch polymers, 2: O-linked profile of human recombinant EPO, 3: di-sialylated Gal-GalNAc standard, 4: partial neuraminidase digest of di-sialylated Gal-GalNAc standard, 5: isolated band 1, 6: band 1+neuraminidase, 7: Gal-GalNAc standard, 8: isolated band 3.

Table 3. Composition of O-linked oligosaccharides of human recombinant EPO. The type of O-linked oligosaccharides were deduced from the results of carbohydrate sequencing

Band No.	Content (%)	Structure
1	52.6	NANA-Gal-[NANA]GalNAc
2	16.6	NANA-Gal-GalNAc
3	30.8	Gal-GalNAc

the rhEPO has a complex type structure (Table 2).

Structure determination of the O-linked oligosaccharide of EPO

The sequences of 3 bands were determined from O-linked oligosaccharides sequencing gel (Fig. 9 & 10).

Band 3 seems to be Gal-GalNAc and band 2 looks like trisaccharide: NANA-Gal-GalNAc. Band 1 is a tetrasaccharide whose structure is NANA-Gal-[NANA-] GalNAc. In contrast to N-linked oligosaccharides, O-linked carbohydrate contains a simple structure. Using peptide specific antibody, it was found that the region 99~129 of an EPO molecule represents the receptor binding region, therefore, glycosylated Ser 126 may be important for receptor binding (Sytkowski and Donahue, 1987).

In vitro bioactivity of EPO was not influenced after removal of the O-linked oligosaccharide (Takeuchi *et al.*, 1989). However, the importance of O-linked oligosaccharide *in vivo* has not been estimated yet.

Glycosylation is one of the most important post-translational modifications which may effect folding, secretion, *in vivo* and *in vitro* activity, and the stability of protein molecules. Both biological activity and the half-life of proteins are influenced by chain branching and sialic acid modification. Depending on the host cell, both factors could be changed. In particular, chain branching may be related to local amino acid sequence or secondary structure, although solid evidence is not available. Further studies are necessary to identify the elements which determine the host-cell dependency, chain branching, and the heterogeneity of oligosaccharide chains.

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