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Biological and physicochemical characterization of recombinant human erythropoietins fractionated by Mono Q column chromatography and their modification with sialyltransferase

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Ten erythropoietin (EPO) fractions differing in sialic acid content, ranging from 9.5 to 13.8 mol mol^{-1} of EPO, were obtained from baby hamster kidney cell-derived recombinant human EPO by Mono Q column chromatography. The mean pI values of the EPO fractions determined by IEF-gel electrophoresis systematically shifted from 4.11 to 3.31, coinciding with the sialic acid content, without a change in the constitution of asialo N-linked oligosaccharides of each fraction. Although a linear relationship between the *in vivo* bioactivity and the sialic acid content of the fractionated samples was observed until 12.1 mol mol^{-1} of EPO, there was no further increase in their activity over 12.4 mol mol^{-1} of EPO. On the other hand, an inverse relationship between the *in vitro* bioactivity and sialic acid content of EPO was observed. Also, we showed that the *in vivo* bioactivity of some fractions with low sialic acid contents was increased after treatment with α2,6-sialyltransferase, but the *in vivo* bioactivity of the other fractions with high sialic acid contents was either decreased or not affected.

Keywords: erythropoietin, BHK cell, Mono Q column chromatography, IEF-gel electrophoresis, pI value, sialic acid, oligosaccharide chain, *in vivo* activity, *in vitro* activity, α2,6-sialyltransferase.

Abbreviations: EPO, erythropoietin; rHuEPO, recombinant human erythropoietin; hCG, human chorionic gonadotropin; BHK, baby hamster kidney; CHO, Chinese hamster ovary; NeuAc, *N*-acetyl neuraminic acid; Gal, galactose; HRCs, hemolyser-resistant cells; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium Na; IEF, isoelectric focusing; pI, isoelectric point.

Introduction

Advances in recombinant DNA technology have led to great development in the manufacture of biopharmaceutical products with cultured animal cells. Many kinds of cytokines have been produced by means of these technologies and the effectiveness of some of them has already been demonstrated in a clinical study [1]. Some of these cytokines are glycoproteins consisting of polypeptide chains and oligosaccharide chains. The important roles of oligosaccharide chains in the biological activity of these cytokines have been reported [2]. The polypeptide chains are strictly determined by their DNA sequence, but the oligosaccharide chains cannot be regulated by the DNA sequence. The structures of oligosaccharide chains are influenced not only by the host-cells, but also by the structure of the polypeptide to which an oligosaccharide

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chain is added [3]. Therefore, these cytokines exhibit microheterogeneity in their oligosaccharide chains, i.e. the cytokines consist of various analogues having microheterogenous carbohydrate structures [1, 4].

To obtain the best quality of biopharmaceutical glycoprotein products, the eligible carbohydrate structures must be determined.

Recombinant human erythropoietin (rHuEPO) is one of the glycoproteins produced in animal cells and about 40% of its molecular mass consists of oligosaccharide chains [5]. The relationship between the oligosaccharide structures and their functions in rHuEPO have been studied in detail [6–13].

In this study, we separated rHuEPO fractions showing microheterogeneity of their oligosaccharide chain structures by Mono Q column chromatography, and attempted the biological and physicochemical characterization of these fractions, including the structures of oligosaccharide chains and sialic acid contents. We also introduced a sialic acid at the non-reducing end of oligosaccharide chains and attempted modification of the rHuEPO fractions. The possibilities of quality improvement of EPO in relation to the sialic acid content are discussed.

Materials and methods

rHuEPO

rHuEPO was purified from the culture supernatant of baby hamster kidney (BHK) cells which had been engineered to produce it [14], according to the method described previously [15]. The purity of rHuEPO was more than 99% when analysed by HPLC on a C4-reverse phase column (The Separation Group, USA).

Fractionation of rHuEPO by anion-exchange Mono Q column chromatography

Eight mg of rHuEPO dissolved in 10 mM sodium phosphate buffer, pH 7.0, was applied to a Mono Q5/5 column (0.5 × 5 cm; Pharmacia, Sweden) equilibrated with 50 mM Tris-Cl buffer, pH 7.2. The column was washed with the same buffer, and then rHuEPO was eluted with a linear gradient of 0–0.3 M NaCl in a total volume of 30 ml at the flow rate of 0.5 ml min^{-1}. Sixty-four mg of rHuEPO, referred to hereafter as 'pre-apply', was fractionated by repeated chromatography, and the corresponding fractions designated as (a) to (j) were combined (Fig. 1). The combined fractions were dialysed against distilled water and then lyophilized. The lyophilized rHuEPO fractions were dissolved in 10 mM sodium phosphate buffer, pH 7.2, and then the protein concentration was determined by the method of Lowry [16] using bovine serum albumin as a standard.

Isoelectric focusing (IEF) gel electrophoresis

Isoelectric focusing gel electrophoresis was carried out on a polyacrylamide slab gel. 5% acrylamide, 0.15% *N,N'*-methylene-bis(acrylamide), 0.15% *N,N,N',N'*-tetramethylenediamine and 0.025% ammonium peroxodisulfate were polymerized, and then electrified at 2000 V for 20 min. Ten μg of each sample, 20 μg of unfractionated rHuEPO, and marker proteins (PI Calibration Kit; Pharmacia, Sweden) were applied on the gel, which was then run for 100 min at 2000 V. After electrophoresis, the gel was

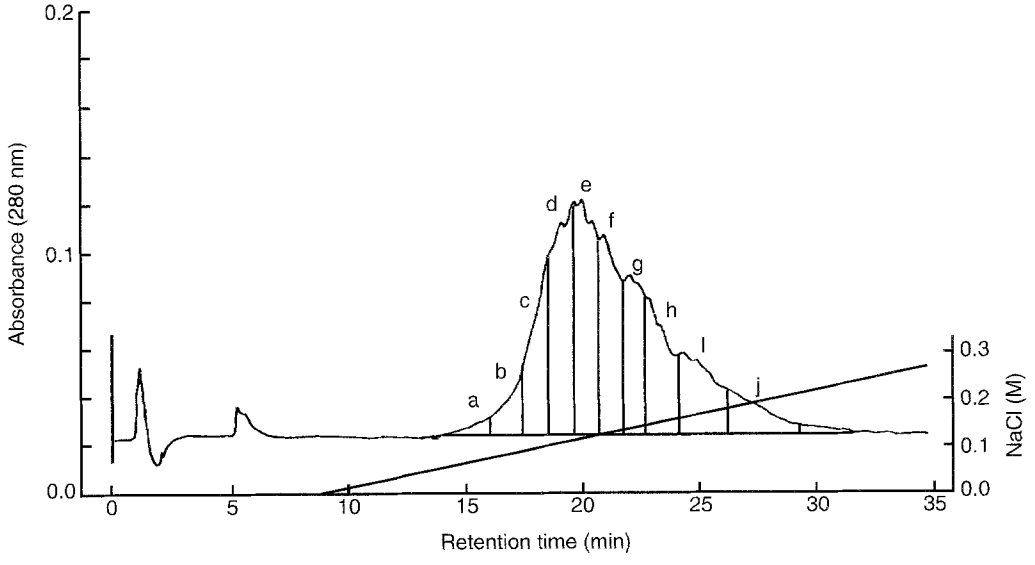

Figure 1. Fractionation pattern of BHK cell-derived rHuEPO on a Mono Q HR 5/5 anion-exchange column. The column was eluted with a linear concentration gradient of 0–0.3 M NaCl in 50 mM Tris-HCl, pH 7.2, at a flow rate of 0.5 ml min^{-1}. Fractions were collected as indicated.

stained with Coomassie Brilliant Blue R-250. The gel was then dried and the stained protein bands on the gel were monitored with a Dual Wavelength Flying Spot Scanning Densitometer CS-9300 PC (Shimadzu, Japan) at the wavelength of 570 nm in the reflection photo mode.

Quantitative analysis of sialic acid

Each concentrated sample of rHuEPO, from (a) to (j), was diluted to 500 μg protein ml^{-1} with 10 mM sodium phosphate buffer, pH 7.2. Ten μl of each sample was digested with 10 mU sialidase from *Streptococcus sp.* (Seikagaku Kogyo, Japan) for 3 h at 37 °C in 25 μl of 100 mM sodium acetate buffer, pH 6.5, containing 10 mM $CaCl_2$. After incubation, the content of released sialic acid was measured by the 2-thiobarbituric acid method [17] using *N*-acetyl neuraminic acid (NeuAc) as a standard.

Structural analysis of asialo N-linked oligosaccharide chains

One mg of 'Pre-apply' EPO and 0.5 mg of samples (b)–(i) were dialysed against water and then lyophilized. The N-linked sugar chains in the EPO samples were released by hydrazinolysis with Hydraclub S-204 (Honen Corporation, Japan). The released N-linked oligosaccharide chains were reductively aminated with 2-aminopyridine [18], and then purified on a TSK-GEL HW 40-F column (TOSOH, Japan) [19]. The labelled N-linked sugar chain samples were dried and then desialylated with 40 mU sialidase from *Streptococcus sp.* (Seikagaku Kogyo, Japan) for 3 h at 37 °C in 100 μl of 100 mM sodium acetate buffer, pH 6.5, containing 10 mM $CaCl_2$. The samples were analysed by HPLC on a reverse-phase ODS-column as described previously [6], with some modifications. The precise conditions were as follows. A TSK-GEL ODS 80 TM column (4.6 × 250 mm; TOSOH, Japan) was used. Elution of each sample was performed at the flow rate of 1.0 ml min^{-1} at 55 °C using two solvents, A and B. Solvent A was 10 mM sodium phosphate buffer, pH 3.8, and solvent B 10 mM sodium phosphate buffer, pH 3.8, containing 0.5% 1-butanol. The column was equilibrated with a mixture of solvents B and A, 18:82 by volume. After injection of a sample, the ratio of solvent B was held at 18% for 20 min, and then increased linearly to 58% in 120 min. In the HPLC system, PA-oligosaccharides were detected as fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. The structure of each oligosaccharide fraction was determined from its retention time on the ODS-column, in comparison with that of previously identified standard oligosaccharides from rHuEPO [6]. The structures of the standard oligosaccharides had already been determined by the two-dimensional mapping technique [20], high-resolution proton nuclear magnetic resonance spectroscopy, and methylation analyses, as described [6].

In vivo *bioassay of rHuEPO*

Female ICR mice, 8–10 weeks old, weighing 20–30 g were used for the *in vivo* bioassay. 0.2 ml of an EPO solution was injected subcutaneously into each mouse once a day for three consecutive days. On day 4, blood was collected and treated as described previously [21, 22]. The count of hemolyzer-resistant cells (HRCs) was taken as an indication of *in vivo* bioactivity.

In vitro *bioassay of rHuEPO*

The *in vitro* bioactivity of EPO was measured as the stimulation of proliferation of the EPO-dependent murine myeloid cell line, EP-FDC-P2, as described in our previous paper [23] with some modifications. Briefly, the cells were maintained in suspension culture in RPMI-1640 supplemented with 10% fetal calf serum and 2 U ml^{-1} of EPO at 37 °C under humid air containing 5% CO_2. For assays, the cells were washed three times with RPMI-1640 supplemented with 10% fetal calf serum, and then re-suspended to a final concentration of 4×10^5 cells ml^{-1} in the same medium. Then, 100 μl of the cell suspension was seeded into each well of a 96-well microplate, followed by culturing for 2 days at 37 °C in the presence or absence of EPO at various concentrations. Cell proliferation was measured by using the water-soluble tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium Na (WST-1) [24] (Dojindo Lab., Kumamoto). After cultivation, 10 μl of a WST-1 assay solution in 10 mM phosphate-buffered saline containing 20 μM 1-methoxy-5-methylphenazinium methylsulfate was added to each well, followed by incubation for an additional 4 h. The absorbance was measured at 405 nm with a microplate reader (Titertek Multiscan Plus, Flow Laboratories). *In vitro* bioactivity was calculated by means of the parallel-line assay from the dose-response curve.

Sialylation of fractionated EPO samples with sialyltransferase

Mono Q-fractionated EPO samples (40 μg) were incubated with 4 mU of α2,6-sialyltransferase (Boehringer Mannheim) and 60 nmol of CMP-*N*-acetylneuraminic acid (Genzyme, Cambridge) in 120 μl of 50 mM sodium cacodylate buffer (pH 6.0) containing 50 mM NaCl at 37 °C for 20–24 h. The same amount of each EPO sample was incubated without α2,6-sialyltransferase under the same conditions and used as a control. Sialylated and control EPO samples without purification were diluted and their *in vivo* bioactivities were examined. The results were statistically analysed by using Student's *t*-test in combination with the *F* test for variability. The extent of sialylation was determined by IEF gel electrophoresis.

Results

Isoelectric focusing (IEF)-gel electrophoresis of fractionated EPO samples by Mono Q column chromatography

BHK cell-derived rHuEPO was separated into ten fractions, samples (a)–(j), by Mono Q column chromatography, as shown in Fig. 1. The IEF-gel electrophoretic patterns obtained, for the fractionated EPO samples were different, as shown in Fig. 2. Although a broad and non-separable smear band, ranging from about 4.3 to 3.0 in isoelectric point (pI), was observed for the pre-apply sample, each fractionated EPO sample gave four to five bands. Table 1 shows the results of densitometric analysis of the bands obtained on IEF-gel electrophoresis of each sample. From the relative ratio of the bands, the mean pI value was calculated. The mean pI values systematically shifted from 4.11 to 3.31.

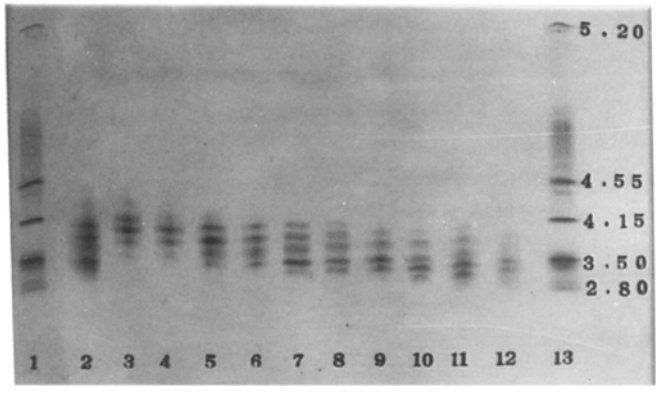

Figure 2. IEF-gel electrophoretic patterns of BHK cell-derived rHuEPO samples obtained by Mono Q column chromatography. Lane 1 and 13 are marker proteins. Lane 2 is pre-apply and lanes 3–12 are samples (a)–(j), respectively.

Structural analysis of oligosaccharide chains of fractionated EPO samples

The sialic acid content of each sample is shown in Table 1. The content varied from 9.53 to 13.80 mol mol^{-1} of EPO, indicating a maximum difference of 4.3 mol mol^{-1} of EPO. The sialic acid contents increased steadily with a decrease in the mean pI value from sample (a) to sample (i); therefore a clear inverse relationship was found between the sialic acid content and the mean pI value, except in the case of sample (j).

To determine the sugar structure, the N-linked oligosaccharide in each sample was prepared by hydrazinolysis. The reducing ends of the oligosaccharide chains obtained were aminated with a fluorescent reagent, 2-aminopyridine, and the mixture of pyridylamino derivatives of the oligosaccharides was analysed by a two-dimensional mapping technique. The assignments of the structures of these oligosaccharides and their relative percentages are summarized in Fig. 3 and Table 2, respectively. Since the quantities of samples (a) and (j) were limited, we could not analyse them further. All of the structures of asialo N-linked oligosaccharide chains in samples (b)–(i) were found to be quite similar.

For example, the oligosaccharide chain of sample (d) consisted of biantennary (3%), triantennary (20%), tetraantennary (53%), and tetraantennary + *N*-acetyllactosamine repeats: one repeat unit (15%), two repeat units (6%), and three repeat units (3%), and thus the tetraantennary structure was dominant. The distribution pattern of these oligosaccharide chains was almost the same for these samples. Therefore it was strongly suggested that the main differences in the sugar chain

Table 1. Distribution of pI values on IEF-gel electrophoresis and contents of NeuAc in each EPO sample obtained by Mono Q column

	Distribution of pI value (%)[a]									*Mean pI*	*Content of NeuAc*[c]
Sample	*4.35*	*4.20*	*4.10*	*3.95*	*3.80*	*3.60*	*3.40*	*3.20*	*3.00*	*value*[b]	*(mol mol^{-1} of EPO)*
a	8	28	42	17	5					4.11	9.53
b		18	41	29	12					4.04	10.28
c		5	21	40	24	10				3.92	11.03
d			16	28	34	22				3.85	11.48
e			11	20	25	35	9			3.75	12.08
f			6	18	27	32	17			3.71	12.38
g				12	22	38	24	4		3.62	12.38
h				6	18	24	34	18		3.52	12.98
i				5	14	18	30	33		3.45	13.80
j					6	14	30	28	22	3.31	13.35

[a]Relative percentage of each EPO band on IEF-gel electrophoresis was measured by densitometer.
[b]Mean pI values were calculated from the distribution pattern of each sample.
[c]Contents of NeuAc were measured by the 2-thiobarbituric acid method.

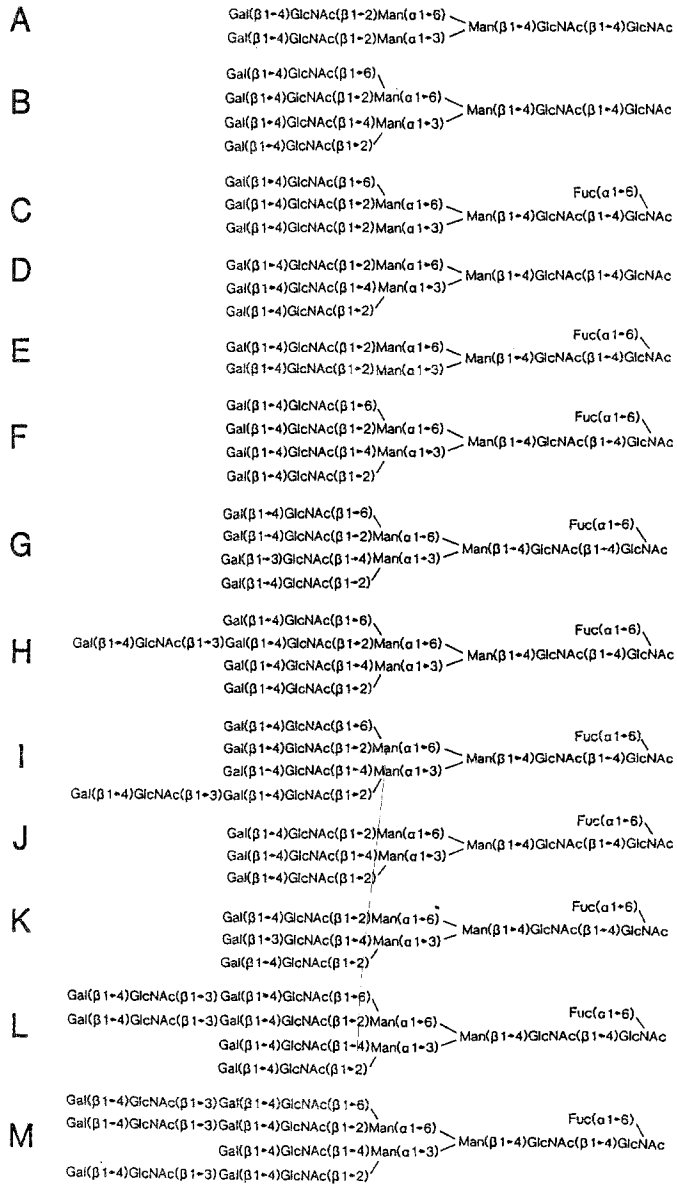

Figure 3. Proposed structure of asialo N-linked oligosaccharides obtained from BHK cell-derived rHuEPO.

structure in each sample resulted from the sialic acid content.

In vivo *and* in vitro *bioactivity of the fractionated EPO samples*

Figure 4 shows the *in vivo* bioactivity of each sample. The activities are expressed as percentages, relative to the pre-apply sample, which was taken as 100%. The activity of the high pI sample with a low sialic acid content obtained from the same host cells under different culture conditions amounted to 94.1%. The relative activity values of EPO in samples (a)–(d) increased from 74.5% to 113.5%. The activities of samples (e) and (f) were about 1.6 times higher than that of sample (a). Although parallel increases in the *in vivo* bioactivity and the sialic acid content were observed from sample (a) to sample (e), there was no further increase in the activity in spite of the increase in the sialic acid content from sample (f) to (j).

Figure 4 also shows the *in vitro* bioactivity of each fractionated EPO sample. The high pI sample and sample (a) showed the highest activity, which was about 120% that of the pre-apply sample, while those of samples (i)

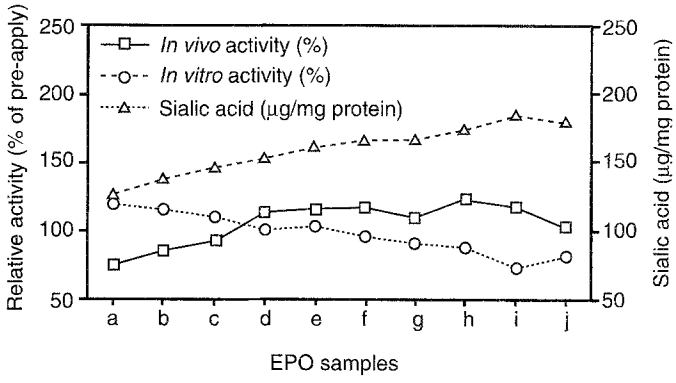

Figure 4. Comparison of *in vivo* and *in vitro* bioactivity of the rHuEPO samples with their sialic acid contents. Values of *in vivo* bioactivity are mean of nine mice. Values of *in vitro* bioactivity are mean of three assays.

Table 2. Asialo N-linked oligosaccharide distributions of EPO samples obtained by Mono Q column

	Amount of sugar chain[a] *in each sample (relative %)*											
Sample	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H,I*	*J*	*K*	*L*	*M*
Pre-apply	1	7	3	3	5	34	10	15	11	4	4	3
b	0	6	3	3	5	32	10	15	10	5	6	5
c	0	6	2	3	2	37	11	18	9	5	5	2
d	0	7	3	3	3	36	10	15	9	5	6	3
e	1	7	2	2	2	47	10	12	7	3	4	3
f	0	7	2	3	3	32	13	14	11	5	5	5
g	1	10	2	3	3	38	10	14	10	3	3	3
h	1	9	2	2	3	36	10	12	11	6	3	5
i	0	10	0	2	4	41	9	15	10	4	3	2

[a]Each sugar structure is shown in Fig. 3.

and (j) were only 70% and 80% that of the pre-apply sample, respectively. From sample (a) to sample (i), the bioactivity decreased from 120% to 73%. A clear inverse relationship between the *in vitro* bioactivity and the sialic acid content was thus observed.

Sialylation of the fractionated EPO samples with sialyltransferase

As shown in Table 3, the *in vivo* bioactivity of samples (a), (b), (c), (d) and (g) increased on sialyltransferase treatment, significantly increasing in samples (a) and (b). On the other hand, decreases in the activity were observed in samples (e) and (f). And in case of sample (h), no change in the activity was observed.

In order to determine the content of sialic acid incorporated into each sample, IEF gel electrophoresis was performed. Table 4 shows the changes in the mean pI values before and after treatment with α2,6-sialyltransferase. The changes in the mean pI values gradually decreased from sample (a) to sample (h). Since the mean pI value exhibited a good inverse relationship with the sialic acid content (Table 1), the incorporated sialic acid was calculated from the changes in mean pI value, as shown in Table 4. The maximum incorporation was observed in sample (a), and the amount of incorporated sialic acid was estimated to be about 1.2 mol mol^{-1} of EPO. The amount of incorporated sialic acid gradually decreased from sample (b) to sample (d). In the case of sample (h), no incorporation was observed.

Discussion

Terminal sialylation of the oligosaccharide chains was found to be an important factor modulating the function of various glycoproteins [1]. Fukuda *et al.* [10] reported the sialic acid residues attached to triantennary and tetra-antennary complex-type oligosaccharides of rHuEPO are indispensable for survival of the EPO in the circulation. Once the sialic acid residues are removed, newly exposed galactose residues are recognized by the galactose binding protein in hepatocytes, and the asialo-EPO is rapidly removed from the circulation.

In the present study, ten fractions differing in sialic acid content, ranging from 9.53 to 13.80 mol mol^{-1} of EPO, were obtained on Mono Q column chromatography. Although rHuEPO gave a single peak on C4-reverse-phase HPLC, EPO was fractionated into samples (a)–(j) on anion-exchange column chromatography. Since the distribution patterns of asialo N-linked oligosaccharides of the samples were similar, Mono Q column chromatography was considered to be an efficient method for separating EPO with different sialic acid contents without a change in the distribution of asialo N-linked oligosaccharide chains.

A linear relationship between the *in vivo* bioactivity and the sialic acid content was observed in the range of 9.5–12.1 mol of sialic acid per mol of EPO. However, there was no further increase in the activity over 12.4 mol of sialic acid per mol of EPO which corresponds to sample (f). The *in vivo* bioactivities of EPO samples together with their sialic acid contents reflected a combined effect of a positive contribution to clearance from the circulation and a negative contribution to the EPO-receptor interaction [7]. Sample (e) or (f) was considered to have the optimum sialic acid content for the *in vivo* biological activity. Therefore, sialic acid contents of around 12.1–12.4 mol mol^{-1} of EPO would be optimum for the *in vivo* bioactivity.

From among the glycoprotein hormones, the biological functions of sialic acid were studied using human chorionic gonadotropin (hCG) [25], EPO [13], and thyrotrophin [26]. But these studies involved the methods of desialylation and resialylation. For example, Imai *et al.*

Table 3. Changes of *in vivo* bioactivity after treatment with sialyltransferase

	In vivo activity[a] *(HRCs × 10^9 per μl)*	
Sample	*Without sialyltransferase*	*With sialyltransferase*
Pre-apply	59.47 ± 10.12	58.17 ± 15.16
High pI	47.30 ± 5.97	66.77 ± 13.49
a	42.93 ± 5.58	64.38 ± 15.82[b]
b	49.16 ± 7.17	74.22 ± 13.20[c]
c	59.18 ± 4.95	71.10 ± 15.61
d	68.98 ± 18.98	73.90 ± 6.87
e	77.00 ± 6.81	74.47 ± 32.88
f	80.67 ± 5.35	70.53 ± 21.40
g	72.57 ± 8.41	84.90 ± 7.06
h	74.17 ± 4.81	74.03 ± 8.05

[a]Data show mean values ± SD ($n = 3$).
[b]$p < 0.05$ from the group without sialyltransferase.
[c]$p < 0.01$ from the group without sialyltransferase.

Table 4. Changes of mean pI values after treatment with sialyltransferase and estimated contents of incorporated NeuAc

	Mean pI values		
Sample	*Before treatment*	*After treatment*	*Incorporated NeuAc*[a] *(mol mol^{-1} of EPO)*
a	4.11	3.99	1.2
b	4.04	3.96	0.5
c	3.92	3.88	0.3
d	3.85	3.82	0.2
e	3.75	3.77	0.1
f	3.71	3.68	0.2
g	3.62	3.58	0.2
h	3.52	3.52	0.0

[a]Contents of incorporated NeuAc were calculated from the differences of mean pI values.

[13] treated rHuEPO from CHO cells with sialidase and then prepared seven kinds of EPO samples differing in sialic acid content ranging from 0.03 to 8.80 mol mol^{-1} of EPO. They concluded that the *in vivo* bioactivity of EPO was fully dependent on the sialic acid content. However, in the present study a different phenomenon was observed as to the sialic acid content ranging from 9.53 to 13.80 mol mol^{-1} of EPO (Fig. 4).

Amano *et al.* [25] reported that an isomeric α2,6-sialylated hCG was prepared from asialo hCG with CMP-NeuAc and α2,6-sialyltransferase, and compared with natural α2,3-sialylated hCG in terms of function. Since both the isomeric hCG and the natural hCG showed similar abilities to produce cAMP, the characteristics of the bond between NeuAc and Gal did not affect the biological activity of hCG. Sasaki *et al.* [9] reported that all N-linked sugar chains in CHO cells are sialylated by α2,3-linkages. The N-linked sugar chains in BHK-21 cell-derived rHuEPO also terminate exclusively in NeuAcα2 $\rightarrow$ 3Gal linkages [8]. Therefore, the NeuAc linkages in the BHK cell-derived rHuEPO might be α2,3-sialylated ones.

In the present study, we tried to introduce sialic acid by using a rat liver α2,6-sialyltransferase. The *in vivo* bioactivities of fractions with low sialic acid contents, such as samples (a) and (b), were significantly enhanced by treatment with the α2,6-sialyltransferase and the fractions also had incorporated sialic acid. On the other hand, sialic acid incorporation was slight or not observed in samples (c)–(h) and the effect of the sialyltransferase treatment on the *in vivo* bioactivity was not significant statistically.

Furthermore, the *in vivo* bioactivities of samples (c)–(f) after sialyltransferase treatment were almost equal to that of sample (b). The bioactivities of samples (a) and (b) after sialyltransferase treatment were higher than that of untreated sample (c) although the mean pI value of untreated sample (c) was lower than those of treated samples (a) and (b) (Tables 3 and 4). The sialic acid content of sample (b) after sialyltransferase treatment was estimated to be 10.8 mol mol^{-1} of EPO. Since the sialic acid content of treated sample (b) was lower than the above mentioned optimum sialic acid content, the sialylation of a specific Gal may be required for maximum EPO activity. This is the first report of the sialylation of an intact glycoprotein with α2,6-sialyltransferase without prior desialylation.

In our previous study [7], the content of O-linked sugar chain, composed of sialic acid and Gal β1 $\rightarrow$ 3GalNac, was found to be approximately 0.85 mol mol^{-1} of EPO at the Ser-126 residue. Wasley *et al.* [27] reported that EPO was produced without O-linked sugar chain by using cells that are deficient in UDP-Gal/UDP-GalNac 4-epimerase activity. Since this EPO exhibits normal *in vivo* bioactivity and *in vivo* clearance, the contribution of O-linked sugar chain to the *in vivo* bioactivity was negligible. Therefore, we discussed only the effects of sialic acid on the N-linked sugar chains of EPO.

From the present study, we concluded that a desirable EPO fraction with an optimum sialic acid content for *in vivo* bioactivity can be obtained by Mono Q column chromatography. Sialic acid can be introduced into some EPO fractions with low sialic acid contents by using sialyltransferase. These approaches will be useful for improving the *in vivo* bioactivity of rHuEPO, and will play a valuable role in the development of pharmaceuticals with desirable oligosaccharide structures and optimal *in vivo* bioactivities.

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