Effects of Site-directed Removal of N-Glycosylation Sites in Human Erythropoietin on Its Production and Biological Properties*  

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Erythropoietin (Epo) has three N-linked sugar chains. Codons for asparagine at N-glycosylation sites in genomic human Epo DNA were replaced with those for glutamine. The wild-type Epo gene and seven mutants that lacked N-glycosylation sites in every possible combination were introduced into baby hamster kidney cells. To study the role of the N-linked sugars in Epo biosynthesis, Epo protein expressed transiently was measured by an enzyme-linked immunosorbent assay. The elimination of all three N-glycosylation sites decreased Epo production to 10% of that of the wild-type Epo. Wild-type and mutant Epos produced by stably transfected cells were partially purified to investigate their properties. Removal of N-glycosylation sites changed affinity of Epo to the receptor. The in vitro activity of Epo that lost all N-glycosylation sites was comparable with that of the wild-type Epo, while the in vivo activity severely decreased. These results indicate that N-linked sugars of Epo have two major functions; N-linked sugars are important for 1) proper biosynthesis and/or secretion and 2) expression of the in vitro activity probably by enhancing survival in the circulation. N-Linked sugars of Epo affect binding affinity of the ligand to the receptor but do not play a key role in expression of the in vitro activity.

glycoproteins are needed for their biosynthesis and secretion, including folding of the protein during translation and protection of the protein from intracellular degradation. Second, carbohydrates confer stability to the protein so that it is not eliminated from the circulation during its travel from the production site to its target cells. Third, carbohydrates are involved in the expression by the glycoprotein of its biological function, including the steps of the binding of the protein to its target cells and intracellular signal transduction.

The removal of terminal sialic acids from Epo destroys its in vivo activity (12–15), and desialylated Epo is cleared from the circulation faster than unmodified Epo (16, 17). In contrast, the in vitro activity of Epo increases with desialylation (8, 13, 14, 18); the increase is probably due to increased affinity to the receptor (8). The function of the core portion of N-linked sugars in the in vitro activity of Epo has not been firmly established. Wojchowski et al. (19) showed that N-glycanase digestion, which removes N-linked sugars from rHuEpo expressed in insect cells, reduced Epo activity. We (8) reported that the in vitro activity of rHuEpo produced in BHK cells was not decreased by complete removal of N-linked sugars with N-glycanase. Takeuchi et al. (18) found that N-glycanase digestion of rHuEpo produced in Chinese hamster ovary cells results in almost complete loss of activity. Recombinant HuEpo in which all N-glycosylation sites have been eliminated by DNA mutagenesis has not been studied. Comparison of mutant Epos with the wild-type Epo may show which of these apparently contradictory results are correct. Prevention of N-glycosylation at desired positions by site-directed mutagenesis and analysis of the in vivo activity of mutant Epos may give us information important for construction of Epo with better therapeutic properties.

By the use of site-directed mutagenesis of HuEpo cDNA, Dube et al. (20) altered the amino acid at each of the carbohydrate attachment sites to prevent glycosylation. Their investigation of the production of mutant Epos indicated that glycosylation of specific sites is critical for proper biosynthesis and secretion of Epo. They found that the in vitro activity was affected by mutation but did not examine the effect of mutation on the in vivo activity.

In this study, we constructed Epo genes mutated by site-directed mutagenesis so that Epos lacking N-linked carbohydrates in every possible combination of the three N-glycosylation sites were produced. Transient production of mutant Epos by BHK cells was examined to find roles of sugars in biosynthesis of Epo. The mutant Epos produced by stably transfected BHK cells were partially purified and their properties, including the biological activity both in vivo and in vitro, were investigated.
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MATERIALS AND METHODS

Oligonucleotide-directed Mutagenesis and Construction of Plasmids—In vitro site-specific mutagenesis was done with an oligonucleotide-directed mutagenesis system (Amersham COT.). The sequence of the nucleotide 5'ACAGTGATTTGCTCATTCA 3' (for mutant NQ2), and 5'GGGAAGATTGGACCAC 3' (for mutant NQ3), were synthesized with an Applied Biosystems 380A DNA synthesizer to replace codons for Asn at positions 24, 38, and 83 of Epo protein with those for Gln, respectively. The nucleotide sequence of the wild-type and mutant Epo genes obtained is shown in Table I. Single-stranded DNA of a plasmid mp18Epo, which consists of M13mp18 and the entire coding sequence of HuEpo genomic DNA, was used as the template for mutation. The mutated single-stranded DNA, mp18EpoNQ1, in which Asn24 was replaced, was used as a template to create mp18EpoNQ12, which contained mutations of both Asn24 and Asn83. Similarly, mp18EpoNQ2 and mp18EpoNQ23 were used as templates for mp18EpoNQ23 and mp18EpoNQ123, respectively. Mutations were checked by dot blot hybridization and DNA sequencing. Mutant HuEpo genomic DNA was inserted into a unique BamHI site of the mammalian expression vector pZIP-NeoSV(X)I (21). The Epo gene in this plasmid is expressed under the control of the promoter in the long terminal repeat of Moloney murine leukemia virus, and this plasmid confers resistance to G418 on the host cells. Nucleotide sequence analysis of the plasmid mp18EpoNQ1, which lost all N-glycosylation sites, was determined to confirm that constructions were correct. The structures of other mutant plasmids were checked by restriction enzyme analysis.

A plasmid, pKSV10CAT, capable of expressing the CAT gene was constructed as follows. The CAT gene fragment from pYEJ001 (Pharmacia LKB Biotechnology Inc.) was inserted into the BglII site of the mammalian expression vector pZIP-NeoSV(X)I (21). The Epo gene in this plasmid is expressed under the control of the promoter in the SV40 early enhancer and promoter. 

Transfection—BHK-21 cells were cultured in Eagle basal medium (GIBCO) containing 10% calf serum (Flow) and 10% tryptose phosphate broth (Difco), unless otherwise specified. A murine cell line, EP-FDC-P2 (22), the growth of which was dependent on Epo or mouse interleukin-3, was maintained in RPMI 1640 medium containing 10% fetal calf serum to remove the in vitro biological activity of Epo. The cells were grown to be nearly confluent in this medium and then the medium was replaced with a low serum medium (3% fetal calf serum). After incubation for 3 days, the CFU-E colonies that were stained for 

Erythropoietin Assay—p1-261 Labeled HuEpo was bound to mouse erythrocytes as described previously (32) with a minor modification. A cell suspension (100 µl) containing 5 × 10⁷ TSAS cells were mixed with 50 µl of PBS containing 60 mM HEPES, pH 7.2, 0.3% bovine serum albumin, 0.3% NaN₃, and 261 labeled rHuEpo (0.5 nM). After incubation for 3 h at 15 °C, the cells were pelleted, washed once with PBS, and suspended in 200 µl of PBS. The suspension was layered on 800 µl of PBS containing 10% bovine serum albumin, and the cells were separated from the unbound ligand by centrifugation. The tube contents were frozen in solid CO₂ / ethanol. The tip was cut off just above the cell pellet, and the radioactivity of the pellet was counted.

N-Glycanase Digestion of Erythropoietin—The wild-type HuEpo was isolated (25). All of N-linked sugars attached to Epo were removed by N-glycanase digestion under the condition as described previously (8). Completion of the digestion was confirmed by altered mobility on SDS-polyacrylamide gel electrophoresis (8).

RESULTS

Effect of N-Glycosylation on Biosynthesis and Secretion of Erythropoietin—The wild-type and mutant Epos were transiently expressed by BHK cells, and the immunoreactive Epo secreted into the culture media was measured by ELISA. Results are summarized in Table I. The production of mutant Epos decreased as the number of N-linked carbohydrate chains was reduced. When all three N-glycosylation sites were eliminated, the transient production of Epo decreased to 10% that of the wild-type Epo, but this level of Epo was still above the limit of detection. Immunoreactive Epo in the culture medium of cells transfected with pKSV10CAT alone or not transfected was negligible. The results suggest that N-glycosylation has profound effects on biosynthesis and/or secretion.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Wild-type and Mutant Erythropoietins—Epo proteins in the culture supernatants of stable transfectants were partially purified by immunoadfinity chromatography and detected by
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TABLE I

Nomenclature of HuEpo constructed in this study and effects of mutation on biosynthesis and secretion of HuEpo in BHK cells

<table>
<thead>
<tr>
<th>Construct</th>
<th>Positions of Asn residues placed to Gln residues</th>
<th>Relative efficiency of HuEpo biosynthesis and secretion (%)</th>
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<tbody>
<tr>
<td>NQ0</td>
<td>Wild-type</td>
<td>100</td>
</tr>
<tr>
<td>NQ1</td>
<td>24</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>NQ2</td>
<td>38</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>NQ3</td>
<td>83</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>NQ12</td>
<td>24 and 38</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>NQ13</td>
<td>24 and 83</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>NQ23</td>
<td>38 and 83</td>
<td>41 ± 7</td>
</tr>
<tr>
<td>NQ123</td>
<td>24, 38, and 83</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

Western blot analysis of Epos.

The wild-type and mutant Epo preparations purified with a monoclonal antibody were separated by SDS-polyacrylamide gel electrophoresis, and Epo proteins were visualized by Western blot analysis. About 10 µg of each protein was used. Lane 1, standard proteins (94,000, phosphorylase b; 69,000, bovine serum albumin; 46,000, ovalbumin; 30,000, carbonic anhydrase; 14,000, lysozyme); lane 2, NQ0; lane 3, NQ1; lane 4, NQ2; lane 5, NQ3; lane 6, NQ12; lane 7, NQ13; lane 8, NQ23; lane 9, NQ123.

Fig. 1. SDS-polyacrylamide gel electrophoresis and Western blot analysis of Epos.

Table 2: In Vitro and In Vivo Activities of Epo Constructs

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Relative biological activity</th>
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<tbody>
<tr>
<td></td>
<td>In vitro (%)</td>
</tr>
<tr>
<td>NQ0</td>
<td>100</td>
</tr>
<tr>
<td>NQ1</td>
<td>180</td>
</tr>
<tr>
<td>NQ2</td>
<td>107</td>
</tr>
<tr>
<td>NQ3</td>
<td>130</td>
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<tr>
<td>NQ12</td>
<td>140</td>
</tr>
<tr>
<td>NQ13</td>
<td>170</td>
</tr>
<tr>
<td>NQ23</td>
<td>66</td>
</tr>
<tr>
<td>NQ123</td>
<td>100</td>
</tr>
</tbody>
</table>

*The Epo preparations containing 10–80 µg of protein were diluted 10-fold by PBS containing rat serum albumin at 2 mg/ml, and 2 ml of the diluted sample was administered to each starved rat. Eight rats were used for each assay. Values were averaged.*
and that of NQ23 was lower. Mutant Epo that had lost an N-glycosylation site at position 24 (NQ1, NQ12, NQ13, and NQ123) had higher activity than their counterparts with the N-linked sugar at position 24 (NQ0, NQ2, NQ3, and NQ23). It seemed that removal of N-glycosylation site at position 24 increases in vitro activity. When culture supernatants of NQ0 and NQ123 were directly assayed by the use of EP-FDC-P2, the Epo in both supernatants had about the same specific activity (data not shown), which agreed with the results obtained with the partially purified Epos (see NQ0 and NQ123 in Table II).

Epo activity of NQ0 and NQ123 was also assayed by the use of stimulation of CPU-E colony formation of fetal mouse liver cells, because this assay evaluated not only growth but also differentiation of erythroid precursor cells responding to Epo. When Epo in the culture supernatant of NQ0 was added in the assay mixtures at concentrations of 0, 0.35, 0.7, and 1.4 pm, numbers of CPU-E colony formed were 36 ± 18, 95 ± 19, 157 ± 28, and 196 ± 24, respectively. The similar results were obtained with the culture supernatant of NQ123 and the partially purified NQ0 and NQ123.

The in vitro activity of the wild-type Epo (NQ0) and mutant Epo (NQ123) that lost all N-glycosylation sites was assayed with human cell line TF-1. The partially purified samples (NQ0 and NQ123) and also the culture supernatants (NQ0* and NQ123*) were assayed. The results are shown in Table III. The activity of NQ123 was somewhat lower than that of NQ0, but the activity of NQ123* was comparable with that of NQ0*.

The activities manifested by all Epos were completely inhibited by the presence of 5 μg/ml monoclonal antibody (25) against rHuEpo. We concluded from these results that N-linked sugars in Epo are not important in the stimulation of cell growth and differentiation in vitro.

Wild-type rHuEpo was isolated and digested with N-glycosidase to remove N-linked sugars. In vitro activity of the Epo that lost all N-linked sugars was compared with that of the undigested Epo, using human cell line TF-1 as target cells. The digested Epo possessed 60–80% of activity of the undigested one.

In Vivo Biological Activity of Wild-type and Mutant Erythropoietins—In vivo activity of NQ1, in which the N-glycosylation site at position 24 was removed, was higher than that of the wild-type Epo, NQ0 (Table II). Mutant Epo (NQ3) that had lost the N-glycosylation site at position 83 had activity similar to that of NQ0, but the activity of NQ2 that lost N-glycosylation site at position 38 was 60% of that of NQ0. The removal of two of the three N-glycosylation sites yielded Epos with about 50% of the activity of NQ0, and the removal of all N-glycosylation sites caused a very large decrease in the activity, yielding Epo with 8% of the activity of NQ0. The control preparations obtained from the culture supernatants of cells that were not transfected did not have any Epo-like activity.

Binding to Erythropoietin Receptor—The enzymatic removal of sugars from Epo by a variety of glycosidases has shown that the carbohydrate chains influence the binding affinity of Epo to the receptor (8); changes in the in vitro activity by the elimination of N-glycosylation sites (Table II) may be caused by changes in the affinity to the receptor. The inhibition of the specific binding of 125I-labeled wild-type Epo to the receptor by unlabeled Epos was investigated (Fig. 3), and the concentrations of Epo needed for 50% inhibition were estimated from the inhibition curves as an index of the affinity of Epo to the receptor (Fig. 3, inset). The affinity increased as the number of N-glycosylation sites was reduced: the wild-type Epo, NQ0, had the lowest affinity, in agreement with the previous conclusion (8) that the presence of carbohydrate chains decreases the affinity of Epo to the receptor. The increase in affinity was large in the mutant Epos (NQ1, NQ12, NQ13, and NQ123) in which the N-glycosylation sites, including position 24, had been removed.

**DISCUSSION**

To investigate roles of three N-linked sugars attached to HeEpo, mutant Epos that lacked N-glycosylation site in every possible combination were produced by BHK cells. Epo production decreased as the number of N-glycosylation sites were reduced, and the removal of all N-glycosylation sites lowered Epo production to 10% of that of the fully glycosylated Epo.

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**TABLE III**

<table>
<thead>
<tr>
<th>Epo</th>
<th>[Epo] (pm)</th>
<th>A&lt;sub&gt;530 nm&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NQ0</td>
<td>0.112 (100)</td>
<td>0.343 (100)</td>
</tr>
<tr>
<td>NQ123</td>
<td>0.079 (69)</td>
<td>0.284 (83)</td>
</tr>
<tr>
<td>NQ0*</td>
<td>0.085 (100)</td>
<td>0.310 (100)</td>
</tr>
<tr>
<td>NQ123*</td>
<td>0.090 (106)</td>
<td>0.288 (93)</td>
</tr>
</tbody>
</table>

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**Fig. 3. Binding of Epos to their receptor.** Binding of 125I-labeled rHuEpo to the receptor was assayed with mouse erythroleukemia cells as described under "Materials and Methods." Inhibition of the binding by unlabeled wild-type Epo (NQ0) and mutant Epos (NQ1–NQ123) was examined to estimate the alteration of their affinity by removal of N-glycosylation sites. The ordinate indicates the binding of 125I-labeled rHuEpo in the presence of unlabeled Epos at concentrations shown in the abscissa. NQ1 (），NQ12（），NQ13（），NQ23（），NQ123（）。 Total binding of 125I-labeled rHuEpo measured in the absence of the unlabeled Epo was 10,270 cpm, and nonspecific binding measured in the presence of 200-fold unlabeled rHuEpo was 400 cpm; the specific binding was 9,870 cpm. Concentrations of HeEpo that gave 50% inhibition of the specific binding were estimated from curves and are shown in the inset.
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NQ0. Dube et al. (20) constructed an expression plasmid containing mutant HuEpo cDNA that had lost all of its N- and O-glycosylation sites and examined the production of the immunoreactive Epo in culture supernatants of stably transfected BHK cells. They found that production is about 0.03% of that of the wild-type Epo. The addition of tunicamycin, an inhibitor of N-glycosylation, to a culture of Chinese hamster ovary cells that had been engineered to produce rHuEpo decreases Epo production to 10% of the drug-free culture (33). These results indicate that full N-glycosylation is important for proper biosynthesis and/or secretion of Epo. Dube et al. (20) reported that the prevention of N-glycosylation at position 38 or 83 impairs production; in our system, the production of the mutant Epos (NQ2 and NQ3 in Table I) was significant (60–78% of that of the wild-type Epo). We examined transient expression, but they used stably transfected cells that were selected on the basis of high level of Epo mRNA. The selected cell lines may not be equivalent in activity of the processes during or after translation including secretion, from which an improper conclusion on function(s) of N-linked sugars of Epo in these processes may be drawn. In this regard, the transient expression system would be more reliable, but it has a drawback in that expression of the cotransfected gene must be measured to normalize variable efficiency of transfection.

A recent report by Takeuchi et al. (18) showed that the removal of N-linked sugars from the HuEpo by N-glycanase causes almost complete loss of the in vitro activity, which contradicts our previous results (8) indicating that the enzymatic removal of N-linked sugars from rHuEpo did not decrease the Epo activity. The result given here for NQ123 that lost all N-glycosylation sites strongly support our previous result. Takeuchi et al. assayed the activity of Epo in terms of its stimulation of growth of human cell line TF-1; in our previous report (8), the activity of Epo was assessed by use of its stimulation of DNA synthesis in fetal mouse liver cells and its stimulation of the CFU-E colony formation of mouse bone marrow cells. The contradictory results might be derived from the different assay methods, including the different target cells. To find if this was the case, we assayed the in vitro activity of the wild-type Epo (NQ0) and NQ123 with human cell line TF-1 as well as murine cells. Furthermore we prepared again rHuEpo that lost all N-linked sugars by N-glycanase digestion, and its in vitro activity was examined with human cell line TF-1. All results of these experiments indicated that N-linked sugars in Epo are not important in expression of the in vitro activity. It appears that the enzymatic removal of N-linked sugars from rHuEpo yields Epo that is temperature-sensitive (8) and that sticks to experimental vessels.

All mutations that removed N-glycosylation sites did not impair binding of Epo to the receptor, and some mutations increased binding affinity to the receptor (Fig. 3); the mutant Epos such as NQ1, NQ12, NQ13, and NQ123 had lost N-glycosylation sites including that at position 24 showed a 2- to 3-fold increase. We have shown that all rHuEpos deglycosylated by glycosidases to various extents bind to the receptor with higher affinity than the undigested Epo (8). The results of both mutated Epo and glycosidase-digested Epo indicate that N-linked sugars are not essential but rather inhibitory for interaction of the ligand with its receptor.

In some mutant Epos (NQ1, NQ12, and NQ13), their in vitro activity (Table II) could be accounted for semiquantitatively by their higher affinity to the receptor. This explanation, however, does not seem to hold true for all mutant Epos. Mutant NQ123 with no N-glycosylation sites had the highest affinity to the receptor, but its in vitro activity was similar to that of the wild-type Epo. Mutant NQ23 had a decreased activity, although its affinity to the receptor was somewhat higher than that of the wild-type Epo. The following two possibilities to cause these results remain to be investigated. One possibility is that N-linked sugars exert some effects on events after binding of the ligand to the receptor. The other possibility is that removal of N-glycosylation sites may impair the stability of Epo; a possible increase in the activity gained by higher affinity to the receptor may be counteracted by loss of the activity during assay. We have shown with glycosidase-digested Epos that carbohydrates attached to Epo contribute greatly to the resistance to heat inactivation (8).

Dube et al. (20) reported that replacement of Asn to Gln at position 24 or 38 gives Epos with the specific activity of about 20% that of the control Epo, but our results showed that removal of each N-glycosylation site (NQ1 and NQ2 in Table II) did not lower the activity; the activity of NQ1 that lost N-glycosylation site at position 24 increased. Dube et al. (20) measured rHuEpo protein in culture medium of transfected cells by competitive radioimmunoassay using a polyvalent anti-rHuEpo antiserum. It may be that the culture medium contains fragments derived from Epo by proteolytic digestion and these fragments are immunoreactive but not biologically active. Their method may detect these fragments and overestimate the antigen, which would give low specific activity of the antigen. Further it is not known if mutant Epos that lost N-glycosylation sites are equivalent to the wild-type Epo in their assay system. For assay of Epo protein, we used a sandwich-type ELISA consisting of two monoclonal antibodies which recognize different epitopes of Epo (25). Thus the incidence that our method detects the fragments derived from Epo is very low, if they are present. It is also noted that we partially purified wild-type and mutant Epos so that these fragments were excluded. It has been confirmed that carbohydrates attached to Epo are not involved in the interaction of these monoclonal antibodies with the antigen (25).

By assaying the in vivo activity of the mutated gene products, we may be able to identify which N-linked chain confers stability of Epo in the circulation and also to find a clue about how to construct Epo with better therapeutic properties, such as higher specific activity and a longer period of time in the circulation. The in vivo activity of HuEpo did not change upon removal of the N-glycosylation site at position 83, and more interestingly, this activity increased when the site at position 24 was eliminated. This increase may reflect higher affinity of the ligand to the receptor (NQ1 in Table II and Fig. 3). Abolishment of two or three sites for N-glycosylation, however, caused a large decrease in the in vivo activity, indicating that N-linked sugars contribute much to the expression of the in vivo activity of Epo by preventing the hormone from being metabolically cleared.

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