Survival of Recombinant Erythropoietin in the Circulation: The Role of Carbohydrates

By Michiko N. Fukuda, Hiroshi Sasaki, Lily Lopez, and Minoru Fukuda

Recombinant human erythropoietin produced in transfected Chinese hamster ovary cells is glycosylated much the same way as the erythropoietin present in human urine. To determine the role of carbohydrates in the stability of recombinant human erythropoietin in vivo, \(^{131}I\)-labeled recombinant erythropoietin was intravenously infused into rats. The erythropoietin was slowly cleared from the blood with a half-life of approximately two hours. Asialoerythropoietin, which was produced by treatment of recombinant human erythropoietin with sialidase, was found to be cleared rapidly from circulation within ten minutes. These data suggest that the galactose binding protein of hepatic cells is involved in the clearance of asialoerythropoietin. Erythropoietin also contains N-glycans with a few N-acetyllactosamine repeats, which can be enriched by tomato lectin affinity chromatography. The lectin-bound fraction was cleared to a larger extent than was the unfraccionated erythropoietin, while the component that did not bind the lectin was found to be stable in the circulation. Authentic N-acetyllactosamine repeats (polylactosaminoglycans) prepared from erythrocytes were similarly rapidly cleared from the circulation to the liver, and this clearance was inhibitable with asialo-\(\alpha\)-acid glycoprotein. These results suggest that (a) the sialic acid of the recombinant erythropoietin is necessary for this glycoprotein hormone to circulate stably and (b) glycoproteins with more than three lactosaminyl repeat units may be cleared by the galactose binding protein of hepatocytes.

Erythropoietin is a glycoprotein hormone with a molecular weight (mol wt) of 31 kilodaltons, with 40% of its molecular size accounted for by carbohydrates. These carbohydrates have been shown to consist of one O-linked (carbohydrate attached through the hydroxyl group of serine or threonine residues) and three N-linked oligosaccharides (carbohydrates attached through the amino group of asparagine residues).\(^1\,2\) Erythropoietin is synthesized in the kidney and circulates in the blood to stimulate red cell proliferation and differentiation in the bone marrow. This hormonal activity is completely dependent on the presence of sialic acid\(^3\) when measured in vivo. But, when the assay is done in vitro, the asialoerythropoietin has been found to have enhanced activity.\(^3\) The apparent loss of activity of the asialoerythropoietin in vivo can be explained by the rapid clearance from the circulation by hepatic cells.\(^3\) When the terminal sialic acid is removed from the oligosaccharide, a galactose residue becomes the new terminal sugar. These galactose-terminated glycoproteins may then be recognized by receptors present on the cell surface of hepatocytes and are internalized by endocytosis, followed by lysosomal digestion.\(^4\) Thus it is apparent that the erythropoietin must be fully glycosylated, including terminal sialic acids, to accomplish its hormone action in vivo.

Human peptide hormones may be produced abundantly by using recombinant DNA expression vectors in cultured cells. Glycoprotein hormones must be expressed in animal cells rather than bacteria or yeast to acquire proper carbohydrate structures. In animal cells transfected with an expression vector containing a cDNA copy of the erythropoietin gene, the polypeptide produced will be subsequently modified by the glycosyltransferases present in the host cells.\(^7\,8\) The therapeutic use of recombinant glycoprotein hormones will depend on their mimicking the actions of naturally produced hormones, including the rate of clearance from the circulatory system. We have previously described the carbohydrate structure of erythropoietin produced in Chinese hamster ovary cells that were transfected with a human erythropoietin cDNA.\(^1\) Recently, Takeuchi et al also reported the carbohydrate structure of recombinant erythropoietin.\(^9\) Both studies showed that the major carbohydrate units of the erythropoietin are sialylated tetraantennary saccharides and that a portion contain N-acetyllactosamine repeats.\(^1,9\) The data presented here describe the role of these carbohydrates, particularly the sialic acid and N-acetyllactosamine motifs, with respect to the survival of the recombinant erythropoietin in the circulation.

MATERIALS AND METHODS

Erythropoietin. Chinese hamster ovary cells were transfected with an expression vector that contains the human erythropoietin cDNA.\(^1\) Erythropoietin was purified from the spent medium of those cells as described previously.\(^1\) The purification procedure was modified from that of Miyake et al,\(^10\) and fractionation on a reverse-phase high-performance liquid chromatograph column was included.\(^1\) Erythropoietin was also purified from the urine of aplastic anemia patients according to Miyake et al,\(^10\) with a similar modification. These erythropoietin samples were prepared by Chugai Pharmaceutical, Ltd (Tokyo).

Polylactosaminoglycans. Branched polylactosaminoglycan peptides were isolated from human adult erythrocyte membranes as described previously.\(^11\) Linear polylactosaminoglycan peptides were purified from human newborn (umbilical cord blood) erythrocyte membranes.\(^11\) Both preparations were labeled with \(^3\)H by the galactose oxidase/NaB\(^{3}\)H\(_4\) method as described previously.\(^12\)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gel electrophoresis was carried out according to Laemmli.\(^13\)
Autoradiography was performed by exposing dried gels to x-ray film (XAR-5, Kodak, Rochester, NY) for several hours at −100°C with an intensifying screen.

Clearance of erythropoietin. A male Sprague-Dawley rat (275 to 300 g) was anesthetized by exposure to an atmosphere of metoxyflurane (Pitman-Moore, Washington Crossing, NJ). One to 3 pmol of [125I] erythropoietin (specific activity, ~3 to 10 × 10⁶ cpm/pmol) was prepared by the chloramine-T method and injected through the vein at the penis. After injection, the tip of the tail was removed, and blood was collected into heparinized tubes at various intervals. The radioactivity of the collected blood was monitored by a gamma counter. At the end of the experiment (30 minutes or three hours after the injection) the rat was killed by an overdose of metoxyflurane. The organs were isolated, and the distribution of radioactivity in the various organs was measured.

Clearance of polylactosaminoglycans. [3H]-labeled erythrocyte polylactosaminoglycans were injected into the rat in the same manner as described earlier. The radioactivity of blood was counted in a scintillation counter.

Sialidase treatment of erythropoietin. [125I] erythropoietin was dissolved in 50 μL of 0.02 mol/L Na-phosphate buffer, pH 7.0, containing 0.15 mol/L NaCl and 1% bovine serum albumin. To this solution sialidase (5 mU) from Clostridium perfringens (type VIII, Sigma Chemical Co, St Louis) was added, and the enzyme reaction was allowed to continue at 37°C for 20 hours.

Fractionation of [125I] erythropoietin by tomato lectin and Datura stramonium agglutinin. [125I] erythropoietin (1 × 10⁶ cpm/10 pmol) was added to 40 μL of a suspension (gel-buffer, 1:1, vol/vol) of tomato lectin–Sepharose 4B in 0.05 mol/L Tris-HCl buffer, pH 7.5. After incubation at 4°C for four hours the mixture was centrifuged, and the supernatant was saved as the unbound fraction. After washing the lectin-Sepharose gel with the same buffer four times, 1 mL of 0.1 mol/L of chitobiose dissolved in the same buffer was added to the gel. Bound [125I] erythropoietin that was eluted with chitobiose was saved as the tomato lectin–bound fraction. The ratio of the radioactivity in the tomato lectin–unbound fraction and in the bound fraction was approximately 7 to 1. The [125I] erythropoietin was also fractionated into bound and unbound fractions by using D stramonium agglutinin Sepharose (E-Y Laboratory, San Mateo, CA) in the same manner as fractionation by tomato lectin.

Preparation of asialo-α-carboxylic protein. α1-Acid glycoprotein from human serum was purchased from Sigma. The α1-acid glycoprotein was treated with 0.01 N HCl at 90°C for one hour to remove sialic acids and was dialyzed against water extensively. This acid-treated α1-acid glycoprotein sample is termed here as asialo-α1-acid glycoprotein.

RESULTS

Clearance of recombinant erythropoietin from the plasma circulation of rats. Recombinant erythropoietin produced in Chinese hamster ovary cells was labeled with [125I] and injected into rats intravenously (IV). A portion of the intact erythropoietin was cleared rapidly, but this apparent clearance is most likely due to specific binding of erythropoietin to bone marrow cells since this apparent early-phase clearance disappears when a larger quantity (more than 4 pmol) of erythropoietin is injected (data not shown). This data suggest that the bone marrow cells are undersaturated with erythropoietin and a part of the exogenous erythropoietin is quickly taken up by bone marrow cells. The majority of the recombinant erythropoietin remained in circulation relatively stable, with 30 minutes (Fig 1).

To determine the role of terminal sialic acid in the clearance of recombinant erythropoietin, similar experiments were performed. The terminal sialic acid residues were enzymatically removed from the recombinant erythropoietin. In contrast to the intact erythropoietin, almost all recombinant asialoerythropoietin rapidly disappeared from plasma within 6 minutes. It was noticed that between 10 and 30 minutes the radioactivity in plasma increased gradually (Fig 1). However, as described later, this apparent reappearance of radioactivity was probably due to the degradation of the erythropoietin. Similar clearance curves were obtained when authentic human urinary erythropoietin and its asialo form were examined (data not shown).

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of plasma components collected during clearance experiments indicated that the recombinant erythropoietin remained intact up to 30 minutes (Fig 2A). On the other hand, the asialoerythropoietin (Fig 2B) may be seen to disappear quickly within six minutes of injection. No increase in specific polypeptide bands is observed that corresponds to the increase in total serum radioactivity found between the ten- and 30-minute time points, which suggests that the increase in radioactivity was due to a form undetectable by gel electrophoresis.

To investigate this possibility, serum samples of labeled asialoerythropoietin collected one and 30 minutes after injection were prepared by the chloramine-T method and injected through the vein at the penis. After injection, the tip of the tail was removed, and blood was collected into heparinized tubes at various intervals. The radioactivity of the collected blood was monitored by a gamma counter. At the end of the experiment (30 minutes or three hours after the injection) the rat was killed by an overdose of metoxyflurane. The organs were isolated, and the distribution of radioactivity in the various organs was measured.

Clearance of polylactosaminoglycans. [3H]-labeled erythrocyte polylactosaminoglycans were injected into the rat in the same manner as described earlier. The radioactivity of blood was counted in a scintillation counter.

Sialidase treatment of erythropoietin. [125I] erythropoietin was dissolved in 50 μL of 0.02 mol/L Na-phosphate buffer, pH 7.0, containing 0.15 mol/L NaCl and 1% bovine serum albumin. To this solution sialidase (5 mU) from Clostridium perfringens (type VIII, Sigma Chemical Co, St Louis) was added, and the enzyme reaction was allowed to continue at 37°C for 20 hours.

Fractionation of [125I] erythropoietin by tomato lectin and Datura stramonium agglutinin. [125I] erythropoietin (1 × 10⁶ cpm/10 pmol) was added to 40 μL of a suspension (gel-buffer, 1:1, vol/vol) of tomato lectin–Sepharose 4B in 0.05 mol/L Tris-HCl buffer, pH 7.5. After incubation at 4°C for four hours the mixture was centrifuged, and the supernatant was saved as the unbound fraction. After washing the lectin-Sepharose gel with the same buffer four times, 1 mL of 0.1 mol/L of chitobiose dissolved in the same buffer was added to the gel. Bound [125I] erythropoietin that was eluted with chitobiose was saved as the tomato lectin–bound fraction. The ratio of the radioactivity in the tomato lectin–unbound fraction and in the bound fraction was approximately 7 to 1. The [125I] erythropoietin was also fractionated into bound and unbound fractions by using D stramonium agglutinin Sepharose (E-Y Laboratory, San Mateo, CA) in the same manner as fractionation by tomato lectin.

Preparation of asialo-α-carboxylic protein. α1-Acid glycoprotein from human serum was purchased from Sigma. The α1-acid glycoprotein was treated with 0.01 N HCl at 90°C for one hour to remove sialic acids and was dialyzed against water extensively. This acid-treated α1-acid glycoprotein sample is termed here as asialo-α1-acid glycoprotein.
Fig 2. SDS–polyacrylamide gel electrophoresis of intact and desialyzed recombinant erythropoietin recovered from rat plasma. Plasma was applied to an SDS–polyacrylamide gel (14% acrylamide) and the proteins separated by electrophoresis. [3H]-labeled proteins were detected by autoradiography. (A) Intact erythropoietin. A labeled component at a mol wt of 66 kilodaltons is a dimer of erythropoietin. (B) Desialyzed erythropoietin. The numbers at the bottom of each lane show the time (minutes) when plasma was taken from the rat.

tion were analyzed by Sephadex G-50 gel chromatography. The majority of radioactivity from the sample collected one minute after injection was eluted in the void volume (data not shown), as would be expected for an undegraded glycoprotein. The sample collected 30 minutes after injection was eluted at the same column volume from the column, thus indicating that these radioactive species were of much lower mol wt. It is probable that the radioactivity increase after ten minutes that is seen in the asialo form of erythropoietin is due to the release of degraded products by cells and that these products are too small to be detected by SDS–polyacrylamide gel analysis.

To determine the sites of localization of asialoerythropoietin uptake, the radioactivity present in the four major organs of the rat were analyzed 30 minutes after injection. The distribution of radioactivity was as follows: liver, 85%; kidney, 9%; lung, 4%; and spleen, 2%. The predominance of asialoerythropoietin in the liver strongly suggests the involvement of receptor-mediated endocytosis specific to galactose in clearance. The clearance of serum glycoproteins has been well documented, and it has been shown that asialoglycoproteins or galactose-terminated glycoproteins are readily bound to galactose binding proteins of hepatocytes and are quickly cleared from circulation.

Intact recombinant erythropoietin is relatively stable in circulation after an initial drop in concentration (Fig 1). To determine the time required for clearance of the recombinant hormone, serum from rats injected with [125I]-labeled glycoprotein was collected for longer times, and the radioactivity present in circulation was analyzed by a gamma counter. Figure 3 shows the clearance of the intact recombinant erythropoietin in rats up to 72 hours. A two-phase clearance curve was obtained, as with the previous studies. An initial rapid α-phase with a half-life (t1/2) of ten minutes was followed by a longer β-phase with a t1/2 of 108 minutes.

The distribution of radioactivity in organs was also determined. At 30 minutes after injection the relative amounts were as follows: liver, 64%; kidney, 24%; lung, 9%; and spleen, 3%. The distribution of the radioactivity at three hours was as follows: liver, 53%; kidney, 39%; lung 6%; and spleen, 2%. After three hours, the erythropoietin located in the kidney was found to be significantly increased, and this label could well be excreted (urinary) material. It is most likely that the intact erythropoietin taken up by the liver contains less sialic acid or more N-acetyllactosamine repeats (see the next section) than does the erythropoietin that remains in the circulation.

**The effect of N-acetyllactosamine repeats on the clearance of erythropoietin.** The majority of recombinant erythropoietin in carbohydrates are tetraantennary saccharides. N-acetyllactosamine repeats are attached to the tetraantennary core saccharide. Oligosaccharides with one lactosamine repeat account for 32.1%, those with two lactosamine repeats account for 16.5%, and those with three

Fig 3. Clearance of intact recombinant erythropoietin from plasma circulation over a period of 72 hours. Results obtained for six rats are presented.
lactosamine repeats are 4.7% of the total saccharides. To investigate the stability of erythropoietin that has N-acetyllactosamine repeats, we fractionated [125I] erythropoietin by using tomato lectin-Sepharose, which binds carbohydrates with more than three N-acetyllactosamine repeats. Experiments similar to those just described were performed by using the tomato lectin-bound and -unbound fractions. Data presented (Fig 4A) demonstrate that the tomato lectin-bound (containing lactosamine repeats) erythropoietin was cleared more rapidly and to a greater extent than was the unbound fraction (Fig 4A). The distribution of the radioactivity in the organs 30 minutes after injection of the bound fraction was as follows: liver, 67%; kidney, 18%; lung 10%; and spleen, 5%. The liver may be seen again to be the major organ for removing erythropoietin from the serum.

Erythropoietin was also fractionated by Datura lectin, which has affinity to the side chain of R → GlcNAcβ1 → 6Man of tetrantennary carbohydrate structures. There was no difference detected in the clearance pattern between Datura-bound and -unbound components of recombinant erythropoietin (Fig 4B).

These results suggest that the length of the lactosamine repeats determines the rate of clearance of sialic acid-containing erythropoietin. To determine whether this is a general recognition signal, polylactosaminoglycans isolated from erythrocytes, which have been identified as containing a biantennary core structure and polylactosaminyl side chains, were therefore tested. As shown in Fig 5 these glycosylated peptides were rapidly cleared from the plasma circulation, and more than 90% of the radioactivity was found to be taken up in the liver. This clearance pattern was identical regardless of whether the erythrocyte polylactosamines were intact or desialylated or whether they were linear or branched (data not shown).

To test whether there was a specific interaction of polylactosamine with a receptor, competitors were added to the assay. The addition of lactose had no effect on the clearance, but asialo-α1-acid glycoprotein released the polylactosamines in the early stage of uptake (Fig 5). This effect is probably due to the replacement of cell surface-bound polylactosamine with asialo-α1-acid glycoprotein. Thus the polylactosaminoglycans and the asialo-glycoproteins appear to be cleared through the same galactose binding receptor in the liver.

Small amounts of one lactosamine repeat have been shown to be present in α1-acid glycoproteins, but intact α1-acid glycoprotein does not compete with erythrocyte polylactosamines for clearance (data not shown). This result suggests that the lactosamine repeat in intact α1-acid glycoprotein is not enough to release erythrocyte polylactosaminoglycans from hepatocytes but that the galactose terminal residue exposed by desialylation of α1-acid glycoprotein can compete with the polylactosamines. These observations suggest that a glycoprotein with a number of lactosaminyl repeats may also be recognized by hepatocytes through the galactose binding protein, even when the glycoprotein is sialylated.

**DISCUSSION**

The present study demonstrates the necessity of sialylated N-glycan modification of erythropoietin for it to achieve its...
hormone activity in vivo. The data presented here showed that the carbohydrates are required for the survival of recombinant erythropoietin in plasma. Our results also show that N-acetyllactosamine repeats, if they are longer than three repeating units, are subject to rapid clearance by the galactose binding receptor of the liver even if terminal sialic acid residues are present. The carbohydrate structures of erythropoietin in relation to their clearance are summarized in Fig 6.

It is well-established that sialic acid residues attached to triantennary and tetraantennary complex-type oligosaccharides are indispensable for the survival of glycoproteins in the circulation. Thus, once the sialic acid is removed, newly exposed galactose residues are recognized by the galactose binding protein in the hepatocytes, and the asialoglycoproteins are rapidly removed from circulation and internalized by hepatocytes. Structural analysis of recombinant erythropoietin has shown that it contains mostly triantennary and tetraantennary complex-type oligosaccharides, and the present study confirms that the sialic acid attached to the erythropoietin polypeptide plays a protective role conducive for the survival of recombinant erythropoietin in circulation.

The carbohydrate analysis of the recombinant erythropoietin showed that the erythropoietin produced by the Chinese hamster ovary cells contains N-acetyllactosamine repeats. Although most of these N-acetyllactosamines are short, with one additional N-acetyllactosamine unit (Galβ1 → 4GlcNAcβ1 → 3), we could detect up to three N-acetyllactosamine repeats on a portion of the recombinant glycoprotein by fast atom bombardment analysis. The erythropoietin molecules containing three N-acetyllactosamine repeats were selected by their preferential binding to tomato lectin, and those molecules were rapidly removed from the circulation by receptors in the liver. We have shown in this study that polylactosaminoglycans generally are rapidly cleared from the circulation by the galactose binding receptor in the liver. This indicates that glycoproteins with longer lactosaminyl repeats are not stable once they are released to plasma.

The behavior of polylactosamines in plasma circulation is consistent with the fact that polylactosamines are abundant in plasma membranes but rare in serum. As exemplified by the band 3 glycoprotein in erythrocyte membranes, the polylactosaminoglycans are major glycoconjugates in some cells in the human body. Little is known, however, about the catabolism of polylactosamines. The present results suggest that polylactosamines could be rapidly internalized by hepatocytes if they were released to plasma. The presence of keratan sulfate, a glycosaminoglycan consisting of sulfated polylactosamines, in human serum suggests that the sulfation at C-6 on galactose residues protects the polylactosaminoglycans from rapid clearance by the galactose binding receptor.
ERYTHROPOIETIN CARBOHYDRATES

The correct form to escape detection and removal by galactose binding receptors of hepatocytes. The determination of the correctly glycosylated forms of potential recombinant therapeutic agents and selection of proper expression vectors and hosts to elicit those structures may be very important in future studies.

ACKNOWLEDGMENT

The authors thank Dr T. Kawaguchi of Chugai Pharmaceuticals for his helpful discussions. We also thank Dr V. Piller-Michel for providing tomato lectin—Sepharose. We are greatly indebted to Dr M. Williams for the critical reading and grammatical improvement of the manuscript.

REFERENCES

Survival of recombinant erythropoietin in the circulation: the role of carbohydrates

MN Fukuda, H Sasaki, L Lopez and M Fukuda