ERYTHROPOIETIN MESSENGER RNA EXPRESSION IN THE KIDNEYS OF MICE WITH ACUTE ANEMIA

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Abstract: Localization of erythropoietin messenger RNA was studied by an in situ hybridization procedure using anti-sense oligonucleotide probes of murine erythropoietin in the kidneys of mice with acute anemia. Acute anemia was induced by depletion of blood. Five hundred microliters of blood were withdrawn from orbital plexus 6, 24, and 30 hours before necropsy. Animals were sacrificed 6 hours after the last bleeding. Erythropoietin levels in the plasma, renal cortex, and medulla were 797.0 mU/ml, 216.7, and 11.3 mU/g protein, respectively, and these values were 50, 16, and 4.5 times higher than those in the untreated control mice. Silver grains indicating erythropoietin messenger RNA expression were observed in the interstitial cells of the renal cortex and outer stripe of the outer medulla in the mice with acute anemia, but not in the renal tubules or glomeruli. Silver grains were not detected in the kidneys of the untreated control mice. From these results, it was concluded that the main area of erythropoietin synthesis in the kidneys was the interstitial cells in the renal cortex and outer stripe of the outer medulla in the mice with acute anemia. (J Toxicol Pathol 9: 51-56, 1996)

Key words: Erythropoietin, Acute anemia, Oligonucleotide probe, In situ hybridization, Mouse

Introduction

Erythropoietin is a glycoprotein which regulates the proliferation or differentiation of erythroid progenitor cells in the hematopoietic organs. The main source of erythropoietin is considered to be the kidneys and liver in adult mammals. However, it is not exactly clear which cells produce erythropoietin, although the cloning of erythropoietin genes has been accomplished. Immunohistochemical studies have revealed that erythropoietin is localized in the glomerular epithelial cells in the kidneys. In addition, erythropoietin-like activity has been detected biochemically in the supernatant from the homogenate of the cultured glomerular, mesangial or tubular epithelial cells. Recently, erythropoietin gene expression has been reported in the peritubular cells in the kidneys using an in situ hybridization procedure. However, it has also been reported that erythropoietin gene expression can be found in the renal tubular epithelial cells using a similar procedure.

It is well known that plasma erythropoietin levels increase dramatically under experimentally induced severe anemia and hypoxia. Although the increase in the plasma erythropoietin level is considered to be caused by increased synthesis of erythropoietin in the kidneys, the exact origin of the erythropoietin remains unclear: the tubular epithelial cells, peritubular cells, or other cells.

In the present study, the main area of erythropoietin synthesis in the kidneys was studied using an in situ hybridization procedure and anti-sense oligonucleotide probes in mice with acute anemia.

Materials and Methods

Animals and treatment

Ten male ICR mice were obtained from a commercial breeding colony (CLEA Japan Inc., Tokyo) at 5 weeks of age and were acclimatized to the environmental conditions for 2 weeks. Acute anemia was induced in half of these mice, and the remaining animals were used as untreated controls. The procedure used to induce acute anemia was a modified version of a previously reported procedure. Five hundred microliters of blood were taken from
the orbital plexus under ether anesthesia 6, 24, and 30 hours before necropsy, and the same volume of saline was given intraperitoneally each time.

_Hematology values and plasma and kidney erythropoietin concentration_

At necropsy, blood samples were taken from an abdominal vein with heparinized syringes under ether anesthesia. Part of the blood was used for hematology. The following values were measured or calculated with an automated hematology analyzer (E-4000 and R-1000, Towa Medical Industries): erythrocyte count, hemoglobin concentration, hematocrit value, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, leukocyte count, and reticulocyte count. The remainder of the blood was centrifuged at 1,500×g for 10 min to obtain the plasma sample for each animal. After exsanguination under anesthesia, both kidneys were removed from each animal. One kidney was divided into cortex and medulla, and each piece was individually homogenized in physiological saline at 4°C. Each homogenized sample was centrifuged at 9,500×g for 30 min at 4°C, and then the supernatant was collected. Erythropoietin concentration in the plasma and supernatant samples was measured by an RIA method. The remaining kidney was used for _in situ_ hybridization.

_In situ hybridization_

Three mouse erythropoietin oligonucleotides probes (EPO 1, EPO 2, and EPO 3) from exon II, III, and V were used as shown in Table 1 in accordance with the mouse erythropoietin gene sequences elucidated by McDonald, J.D. et al.11. Each antisense probe was a 45-mer oligonucleotide, and the 3’-terminal was labeled with 35S-dATP. A cocktail of the three probes was used on frozen kidney sections in accordance with the methods in previous reports27,28. Removed kidneys were frozen in liquid nitrogen and cut at a width of 10 μm with a cryostat. Kidney sections were dried at room temperature for 10 min and then were fixed in ice-cold 4% formalin in phosphate-buffered saline (PBS) for 30 min. After being washed with PBS, sections were acetylated with a 0.25% acetic anhydride aqueous solution containing 1.5% triethanolamine and 0.9% NaCl for 10 min. After being dehydrated with a graded ethanol series, sections were immersed in chloroform for 5 min and air-dried. Hybridization was carried out in a moist chamber overnight at 42°C by incubating the sections with the required 35S-labeled probes (EPO 1+EPO 2+EPO 3) in a hybridization buffer composed of 50% formamide, 4×sodium saline citrate (SSC), 0.25 mg of yeast transfer RNA (tRNA)/ml, 0.5 mg of sheared salmon sperm DNA/ml, 10% dextran sulfate, and 20 mM dithiothreitol (DTT). After hybridization, the sections were washed with 4×SSC at 25°C for 3 min and then with 1×SSC at 55°C for 60 min. Sections were dehydrated with a graded ethanol series and air-dried. Sections were then dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled water), exposed for 4 weeks at 4°C, developed, fixed, and stained with hematoxylin. The sections were observed under a light microscope and photographed.

_Statistics_

The data on hematology and erythropoietin concentrations were analyzed statistically as follows. First, the data were tested by the F test for homogeneity of variance between the control and anemic mice29. If the variances were homogeneous, the Student’s t-test was applied, and if the variances were heterogeneous, the Aspin and Welch t test was performed to compare the mean of the control values with that in the anemic mice. All statistical tests were conducted at the 5% and 1% two-tailed probability levels.

| Table 1. Anti-sense DNA Probes Used for Detection of Mouse Erythropoietin mRNA in the Kidneys in Mice with Acute Anemia |
|------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| EPO 1 5’-ATT | TTC  | TGC  | CTC  | CTT  | GGC  | CTC  | TAA  | GAT  | GTA  | CCT  | CTC  | CAG  | AAC  | TCG-3’ | Exon II |
| EPO 2 5’-GGA | GTT  | GGC  | GTA  | GAC  | TGT  | AAT  | ATT  | TTC  | ACT  | CAG  | TCT  | GGG  | ACC  | TCC  | TGC-3’ | Exon III |
| EPO 3 5’-GAA | TAC  | TGG  | GAC  | TGT  | AAT  | ATT  | TTC  | ACT  | CAG  | TCT  | GGG  | ACC  | TCC  | TGC-3’ | Exon V |
Results

**Hematology (Table 2)**

Hematological examination showed that erythrocyte count, hematocrit value, and hemoglobin concentration were decreased significantly and the percentage of reticulocytes was increased significantly in the mice with acute anemia as compared to the values in the untreated control group. No significant changes were observed in the leukocyte count, platelet count, MCV, MCH, or MCHC in the mice with acute anemia.

<table>
<thead>
<tr>
<th>Table 2. Hematological Parameters in Mice with Acute Anemia</th>
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<tbody>
<tr>
<td>Number of animals</td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Erythrocytes (x 10^6/μl)</td>
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<tr>
<td>Hemoglobin (g%)</td>
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<tr>
<td>Hematocrit (%)</td>
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<tr>
<td>MCV (μl)</td>
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<tr>
<td>MCH (pg)</td>
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<tr>
<td>MCHC (%)</td>
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<tr>
<td>Platelets (x 10^6/μl)</td>
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<tr>
<td>Leukocytes (x 10^6/μl)</td>
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<tr>
<td>Reticulocytes (%)</td>
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*: Mean±S.D.
MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, **: Significantly different from the control group, p<0.01

<table>
<thead>
<tr>
<th>Table 3. Plasma and Kidney Erythropoietin Levels in Mice with Acute Anemia</th>
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</thead>
<tbody>
<tr>
<td>Number of animals</td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Plasma (mU/ml)</td>
</tr>
<tr>
<td>Kidney (mU/g protein)</td>
</tr>
<tr>
<td>Cortex</td>
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<tr>
<td>Medulla</td>
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*: Mean±S.D.
*: Significantly different from the control group, p<0.05
**: Significantly different from the control group, p<0.01

Fig. 1. *In situ* hybridization for mouse erythropoietin mRNA using a mixture of probes in the kidneys in mice with acute anemia. The erythropoietin mRNA-positive cells are located in the cortex and outer stripe of the outer medulla but not in the inner medulla. (x28)
Discussion

Erythropoietin mRNA expression was observed in the interstitial cells in the cortex and outer stripe of the outer medulla of the kidneys in the mice with acute anemia in the present study. Therefore, the erythropoietin producing cells in the kidneys were considered to be the interstitial cells in the cortex and outer stripe of the outer medulla. However, erythropoietin producing cells have been identified as the glomerular epithelial cells, mesangial cells, or cortical tubular epithelial cells in the kidneys by immunohistochemical examination. Erythropoietin mRNA expression was observed in the cortical peritubular cells in the kidneys using an in situ hybridization method when erythropoietin synthesis was stimulated by experimentally induced anemia. In those studies, the erythropoietin probe fragment obtained by cutting with the BamH1 and SmaI or EcoRI, 2,125 or 2,783 base pairs were used in an in situ hybridization method. Although anti-sense 45-mer oligonucleotide probes of mouse erythropoietin were used for in situ hybridization of erythropoietin in the present study, erythropoietin mRNA expression was observed in the cortical peritubular cells and considered to be similar to the results shown in previous studies. Therefore, these probes used in the present study were confirmed to be useful for in situ hybridization of erythropoietin.

Plasma and kidney erythropoietin (Table 3)

Erythropoietin levels in the plasma and renal cortex and medulla in the mice with the acute anemia were 797.0 mU/ml, 216.7, and 11.3 mU/g protein, respectively, and these values were 50, 16, and 4.5 times higher than those in the untreated control group.

Erythropoietin mRNA in the kidneys (Table 4, Figs. 1 & 2)

Silver grains indicating erythropoietin mRNA were observed in the interstitial cells in the cortex and outer stripe of the outer medulla in the kidneys of mice with the acute anemia. However, no silver grains were found in the glomeruli or tubular epithelial cells in the same region. In addition, erythropoietin gene expression was not found in the inner medulla or papilla of the kidneys in the mice with acute anemia or in any region of the kidneys in the untreated control mice.
level in the renal cortex in the mice with acute anemia was also increased, and the value was 16 times higher than the control value. Although the erythropoietin level in the renal medulla was 4.5 times higher than the control value, the absolute value (11.3 mU/g protein) was lower than that in the renal cortex (216.7 mU/g protein). Erythropoietin mRNA expression was found in the interstitial cells in the cortex and outer stripe of the outer medulla of the kidneys in the mice with acute anemia. It is considered therefore that the increase in erythropoietin synthesis in the renal cortex was the main reason for the increase in the plasma erythropoietin level.

From the results of our study, it was concluded that the main area of erythropoietin synthesis in the kidneys was the interstitial cells in the renal cortex and outer stripe of the outer medulla in the mice with acute anemia.

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References


