A Novel Site of Erythropoietin Production

OXYGEN-DEPENDENT PRODUCTION IN CULTURED RAT ASTROCYTES*

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EXPERIMENTAL PROCEDURES

Cell Culture—Cerebral cells were cultured according to the method reported (23). Briefly, the brain was removed from Wistar fetal rats on day 18 under sterile conditions and washed three times with PBS to remove the red blood cells. Meninges were carefully removed, and the resulting cerebral tissue was thoroughly washed with PBS. The tissue was dissociated by passing it through a 70-μm nylon mesh (cell strainer, Falcon). The cell suspension was washed twice with Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Whitaker M. A. Bioproduct). The cells were plated with cells from one fetal brain/50 cm² of cell culture vessel. The cell cultures were carried out at 21, 5, or 1% O₂, 5% CO₂, balance N₂. The medium was changed every 3 days. When O₂ tension was shifted in the course of cell culture, cultures were started with the fresh media equilibrated with the desired O₂ tension by prior incubation for 24 h.

Epo Assay with Enzyme-linked Immunoassay—Epo was measured by an improved method of a sandwich-type enzyme-linked immunoassay (24) using two mAbs, R2 and R6, that recognize different epitopes of Epo. This improved method measures Epo as low as 100 microunits/ml (1 pg/ml). The amount of Epo was calculated from the standard curve drawn using recombinant human Epo (25) as the standard, which had been standardized with the Second International Standard Reference Preparation for human Epo.

Purification of Brain and Serum Epo—Cerebral cells were cultured in 175-cm² T-flasks for 30 days in 5% CO₂, and Epo in the spent medium (800 ml containing about 16 ng of Epo) was purified with the gel (600 ml) containing 0.1% BSA. The resulting solution was ultrafiltrated (Molcut L, Millipore) to concentrate up to 500 μl and exchange the buffer with PBS. Rat serum Epo was isolated from anemic animals (26). Epo from both sources was stored in PBS containing 0.1% BSA.

Treatment of Epo with Neuraminidase—For treatment of Epo with neuraminidase, PBS was exchanged with 0.2 M acetate buffer, pH 5.0, using Molcut L Epo (about 16 ng) was incubated with or without 0.5

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1 The abbreviations used are: Epo, erythropoietin; Epo-R, erythropoietin receptor; PBS, 10 mM phosphate-buffered saline, pH 7.4; Epo-R, soluble Epo-R; BSA, bovine serum albumin; mAb, monoclonal antibody; PCR, polymerase chain reaction; bp, base pair(s).


It has been shown that neurons express erythropoietin (Epo) receptor, but the production of Epo protein in neural tissues has not been demonstrated. Cerebral cells of rat fetuses were cultured, and Epo in the spent medium was measured with an enzyme-linked immunoassay. Production of the immunoreactive Epo was dependent on O₂ tension for cell culture; hypoxia enhanced the production. The immunoreactive Epo purified from the spent medium stimulated the growth of Epo-dependent myeloid cells and formation of fetal liver erythroid colonies. These biological activities were completely inhibited by the anti-Epo antiserum and the extracellular domain of the Epo receptor capable of binding with Epo. When brain Epo was compared with serum Epo, brain Epo was smaller in size and more active in vitro at low ligand concentrations. These differences appear to be caused by the different extent of sialylation. Analysed with the reverse transcription-polymerase chain reaction method indicated that the regulation of Epo production by oxygen operates at the level of its mRNA. Immunohistochemical staining of the immortalized clonal cells revealed that astrocytes produced brain Epo. These results provide a novel site of Epo production and suggest that Epo acts on neurons in a paracrine fashion.

Epo is the glycoprotein that plays a major role in the regulation of erythropoiesis by stimulating the proliferation and differentiation of erythroid precursor cells (1, 2). The kidney is the major organ of Epo production in adult (3) whereas the liver is the primary site in fetus (4). Peritubular interstitial cells in the kidney (5, 6) and more recently peritubular interstitial fibroblasts (7) have been shown to express Epo mRNA. In the liver, hepatocytes and also nonparenchymal cells express Epo (8–10). Oxygen controls the Epo production; hypoxic conditions activate expression of the Epo gene in animals (11–15) and Epo-producing hepatic cells (10, 16–18). Epo has been thought to exclusively act on erythroid precursor cells in vivo, but there is growing evidence that neurons express erythropoietin (Epo) receptor, but the production of Epo protein in neural tissues has not been demonstrated. Cerebral cells of rat fetuses were cultured, and Epo in the spent medium was measured with an enzyme-linked immunoassay. Production of the immunoreactive Epo was dependent on O₂ tension for cell culture; hypoxia enhanced the production. The immunoreactive Epo purified from the spent medium stimulated the growth of Epo-dependent myeloid cells and formation of fetal liver erythroid colonies. These biological activities were completely inhibited by the anti-Epo antiserum and the extracellular domain of the Epo receptor capable of binding with Epo. When brain Epo was compared with serum Epo, brain Epo was smaller in size and more active in vitro at low ligand concentrations. These differences appear to be caused by the different extent of sialylation. Analysed with the reverse transcription-polymerase chain reaction method indicated that the regulation of Epo production by oxygen operates at the level of its mRNA. Immunohistochemical staining of the immortalized clonal cells revealed that astrocytes produced brain Epo. These results provide a novel site of Epo production and suggest that Epo acts on neurons in a paracrine fashion.

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Western blotting, it was necessary to concentrate Epo and remove BSA unit of neuraminidase from Arthrobacter ureafaciens (Nacalai Tesque) brought to 500 ml. The reaction mixture was neutralized with NaOH, and the volume was then added for the stabilization of Epo. Epo (16-25 ng in 1.5 ml of PBS containing 0.1% BSA and 0.05% Tween 20) was attached to the gel (5 pl) to which the Epo-directed mAb R6 (24) was fixed. The gel was washed with 1.5 ml of 0.05% Tween 20 in PBS to remove BSA and centrifuged. To the gel suspension (15 pl) was added the sample buffer (20 ml) for SDS-polyacrylamide gel electrophoresis, and the mixture was heated at 100 °C for 5 min to elute the bound Epo. The gel suspension was centrifuged, and the supernatant (20 ml) was heated again after the addition of 1.3 ml of β-mercaptoethanol. After centrifugation, proteins in the supernatant (20 ml) were separated by SDS-polyacrylamide gel electrophoresis (12% acrylamide gel). Separated proteins were transferred to the nitrocellulose filter and detected using the affinity-purified rabbit anti-Epo antibody, anti-rabbit IgG biotinylated antibody (Vector Laboratories, Inc.), avidin-biotin-peroxidase complex (Vector Laboratories, Inc.), and the ECL Western blotting detection system. The purified rabbit anti-Epo antibody was prepared from the anti-Epo antiserum using the recombinant human Epo-fixed gel (24).

Detection of Epo mRNA—Total RNA was prepared from cultured cerebral cells and the kidneys of the anemic rats by the acid guanidinium thiocyanate-phenol-chloroform extraction method (28). Reverse transcription-PCR was performed according to the method described (29). The reverse transcription reaction was done using a random hexamer and 4 μg of heat-denatured RNA in a volume of 20 ml. The reaction mixture without reverse transcriptase or RNA were run as controls. PCR amplification of random hexamer-primed first-strand cDNA was done using 5 μl of reverse transcription mixture in a total volume of 25 μl containing 200 nM primers of cdNA of rat kidney Epo (30) as follows: sense primer (REP161F, 5' -TCTCTGCTACTGATTCCGTTGG-3') and antisense primer (REPS12R, 5'-AAGTATCCGCTGTGAGTGTTCG-3'). Each of the 50 cycles of PCR consisted of incubation for 1 min at 95 °C for denaturation, 2 min at 65 °C for annealing, and 3 min at 72 °C for elongation. For PCR amplification of β-actin, the sense primer (5' -TCTATGGGTGGCAGCAGGC-3') and antisense primer (5'-TACATGCCTTGGCTGGTTGCTG-3') were used.

RESULTS

Oxygen-dependent Production of Epo by Cultured Rat Brain Cells—Cerebral cells from 18-day-old fetuses of rats were cultured in an O2 tension of 21 and 5%, and Epo in the spent medium was assayed with an enzyme-linked immunosassay (Fig. 1A). When the cells were cultured in 21% O2, Epo production was first detected after 18 days, but its level was low, and this low production continued later. In 5% O2, the high production was observed after 12 days of culture, and hereafter the production increased gradually. To determine whether the regulation of the Epo production by O2 was reversible, the cells cultured for 18 days in 5% O2 were placed in different O2 tensions for 3 days, and then O2 concentrations were changed to 1.5, and 21% at 3-day intervals. The time value at day 18 indicates Epo produced for days 19–18 in 5% O2. In C, cultures were done as in B except that O2 tension was changed to 21, 1.5, and 1% in that order.

Determination of Cellular Protein—The cells were washed thoroughly with PBS and then denatured by adding 1 m of 0.1% HCl. The precipitate was dissolved in 1 ml of 1 N NaOH. Protein in the cell lysates was determined with a protein assay kit (Bio-Rad) using bovine immunoglobulin as a standard.
Brain Erythropoietin

In Vitro Biological Activity of Brain Epo—Brain Epo was partially purified (as described under "Experimental Procedures"), and serum Epo was isolated from anemic rats (27). In A, Epo was assayed by Epo-dependent growth of EP-FDC-P2 cells measuring the increased absorbance at 600 nm due to the preparation that was obtained from the fresh medium by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction (33), and in B, with Epo-dependent erythroid colony formation of fetal mouse liver cells (34). O, brain Epo; A, serum Epo; O, brain Epo in the presence of sEpo-R capable of binding with Epo (35); A, in the absence of sEpo-R. When assayed in the presence of sEpo-R, Epo was preincubated with sEpo-R (100 μg/ml) for 1 h. Each point in A is the mean ± S.D. in quintuplicate, and that in B is the mean in duplicate.

In Vitro Biological Activity of Brain Epo—We partially purified the immunoreactive material (brain Epo) from the spent medium using the mAb-fixed gel. Recovery of the purification was about 90%. There was little immunoreactive Epo in the preparation that was obtained from the fresh medium by the same purification procedure. The purified brain Epo showed dose-dependent biological activities when assayed with two in vitro criteria, stimulation of Epo-dependent cell growth (Fig. 2A) and erythroid colony formation of fetal mouse liver cells (Fig. 2B). The soluble Epo-R (sEpo-R), an extracellular domain of the receptor that is capable of binding with Epo (35), abrogated the biological activities of brain and serum Epo (Fig. 2, A and B) whereas the heat-denatured sEpo-R (5 min at 100 °C) did not (data not shown). These activities were also inhibited by the rabbit anti-Epo antiserum but not by the non-immune rabbit serum (data not shown).

Assays of Epo-dependent cell growth provide more precise data than those of colony formation. When assayed with the cell growth in low concentrations of Epo, brain Epo was more active than the Epo isolated from rat serum (Fig. 2A). Although we have not done the receptor binding assay because it is difficult to prepare brain Epo in a necessary amount, this result indicates that brain Epo binds to Epo-R with an affinity higher than serum Epo. Higher affinity of brain Epo may be attributable to the incomplete sialylation as described below.

Western Blotting of Brain and Serum Epo—Brain Epo migrated on the polyacrylamide gel with a molecular size of 33 kDa, which was smaller than serum Epo (35 kDa) (Fig. 3B, lanes 1 and 2). Treatment of both Epo with neuraminidase at pH 5.0 for 24 h yielded a product with a reduced size of 31 kDa (Fig. 3B, lanes 2 and 4), but the treatment without enzyme did not change the size, which confirmed that the size reduction was due to enzymatic removal of sialic acids (Fig. 3A, lane 1 and Fig. 3B, lane 1; Fig. 3A, lane 2 and Fig. 3B, lane 3). These results indicate that the difference in size between brain and serum Epo is mainly due to poor sialylation of brain Epo. Desialylation yields Epo with higher affinity to Epo-R and consequently an increased in vitro activity (36), which may account for the higher activity of brain Epo compared with serum Epo (see Fig. 2A). The two components with 67 and 55 kDa, which are visible in all samples, are the residual BSA added to stabilize Epo and the heavy chain of the antibody detached from the mAb-fixed gel, respectively. These components are present in the control sample (PBS containing 0.1% BSA), which was treated with the same procedures as the Epo preparations (Fig. 3A, lane 3).

Oxygen-dependent Expression of Epo mRNA—We next detected brain Epo mRNA by the reverse transcription-PCR method. Cerebral cells were cultured in 21 and 1% O2 (three dishes for each O2 tension), and RNA isolated from the individual dishes was used for reverse transcription-PCR. Staining of amplified DNA with ethidium bromide revealed a sharp band with a size of 451 bp (Fig. 4A), the exact size expected from mRNA of rat kidney Epo (30). The band signal derived from the culture in 1% O2 was approximately 20-fold stronger than that from the culture in 21% O2. Similar findings were obtained when the amplified DNA was detected with Southern blotting using Epo cDNA as a probe (Fig. 4B). A diffuse band with a size of 160 bp (Fig. 4A) is a PCR artifact because this band does not hybridize with the cDNA probe (Fig. 4B). The signal of β-actin mRNA was unchanged at different O2 tensions (Fig. 4A). These restriction fragments of the product derived from cerebral RNA are consistent with those expected from the cDNA of rat kidney Epo (30) and also identical to those of the product from the kidney RNA of rats made anemic for accumulation of Epo mRNA, confirming that the 451-bp band is a specific product of Epo mRNA (Fig. 4C).

Identification of Brain Cells Producing Epo—In our cerebral cell culture (Fig. 1), the cells proliferated during a lag period with an undetectable production of Epo, and the proliferated cells appeared to produce Epo. Since neurons from 18-day-old fetuses of rats do not proliferate, it is unlikely that they produce Epo. Proliferating microglial cells appeared atop the astrocytic layer or floated in the medium, and they could be removed by shaking the culture vessels. The isolated microglial cells were recultured, but the production of Epo was undetectable.

From microscopic inspection, astrocytes appeared to be the major population (>95%) in our cultures. These cells were co-transfected with two plasmids conferring drug resistance and cell immortalization. The colonies resistant to G418 were picked up. The cells propagated from most colonies (95/100) produced Epo. Five colonies producing Epo were randomly selected, and the cells were cloned by limiting dilution. All of the established five clones showed hypoxia-induced production of Epo, but the response to hypoxia as well as the production in normoxia varied from clone to clone (Table I).

The clonal cells were immunochemically stained with the
before Western blotting, the samples (brain Epo, serum Epo, and PBS containing 0.1% BSA) were treated as described under "Experimental Procedures." 

A, Epo stored at pH 7.4. Lane 1, brain Epo (33 kDa); lane 2, serum Epo (35 kDa); lane 3, PBS containing 0.1% BSA, which was treated with the same procedures as Epo. B, Epo incubated with or without neuraminidase at pH 5.0 for 24 h at 37°C. Lane 1, brain Epo (35 kDa) incubated without neuraminidase; lane 2, brain Epo (31 kDa) incubated with neuraminidase; lane 3, serum Epo (35 kDa) incubated without neuraminidase; lane 4, serum Epo (31 kDa) incubated with neuraminidase. Proteins with 67 and 55 kDa are the residual BSA and heavy chain of IgG, respectively.

Fig. 3. Western blotting of brain and serum Epo. Epo was stored in PBS containing 0.1% BSA until use. To concentrate Epo and remove BSA before Western blotting, the samples (brain Epo, serum Epo, and PBS containing 0.1% BSA) were treated as described under "Experimental Procedures." A, Epo stored at pH 7.4. Lane 1, brain Epo (33 kDa); lane 2, serum Epo (35 kDa); lane 3, PBS containing 0.1% BSA, which was treated with the same procedures as Epo. B, Epo incubated with or without neuraminidase at pH 5.0 for 24 h at 37°C. Lane 1, brain Epo (35 kDa) incubated without neuraminidase; lane 2, brain Epo (31 kDa) incubated with neuraminidase; lane 3, serum Epo (35 kDa) incubated without neuraminidase; lane 4, serum Epo (31 kDa) incubated with neuraminidase. Proteins with 67 and 55 kDa are the residual BSA and heavy chain of IgG, respectively.

FIG. 4. Detection of Epo mRNA. The cells from rat fetal cerebrum were cultured for 18 days in 5% O2 and then exposed in 21% O2 for 2 days to repress Epo production. Then the cells were cultured for 8 h in 21 and 1% O2 (three dishes for each O2). Epo mRNA in the total RNA from each dish (60 cm2 containing about 10° cells) was amplified with reverse transcription-PCR. In A, the amplified products of cerebral RNA were visualized with agarose gel electrophoresis in the presence of ethidium bromide. M, marker; no, without RNA in the reverse transcriptase (RTase) reaction; +, with reverse transcriptase in the reverse transcription reaction. In B, the amplified products of cerebral RNA were detected by Southern blotting using the 32P-labeled fragment of rat Epo cDNA (30) as a probe. In C, the amplified product of cerebral and kidney RNA was precipitated with polyethylene glycol, digested with SacI, EcoT141, or HindII, and the resulting fragments were detected with Southern blotting. From the sequence of rat Epo cDNA (30), digestion with SacI, EcoT141, and HindII should yield the hybridizable fragment with 377, 356, and 276 bp, respectively. Agarose concentrations were 1% in A and B and 3% in C.

TABLE I

| Table 1 Oxygen-dependent production of Epo by cloned astrocytes |

<table>
<thead>
<tr>
<th>Clones</th>
<th>Epo produced for 24 h</th>
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<tr>
<td></td>
<td>Medium</td>
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<td></td>
<td>21% O2</td>
<td>1% O2</td>
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<tr>
<td>Z1</td>
<td>5.4 ± 0.3</td>
<td>14.8 ± 1.2</td>
<td>3.6 ± 0.1</td>
<td>13.1 ± 1.3</td>
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<tr>
<td>Z2</td>
<td>9.5 ± 0.4</td>
<td>31.2 ± 3.9</td>
<td>4.9 ± 0.2</td>
<td>23.9 ± 2.7</td>
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<tr>
<td>D2</td>
<td>7.8 ± 0.4</td>
<td>18.4 ± 1.2</td>
<td>6.4 ± 0.3</td>
<td>18.6 ± 0.5</td>
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<tr>
<td>D3</td>
<td>9.1 ± 0.5</td>
<td>26.5 ± 2.6</td>
<td>6.5 ± 0.4</td>
<td>24.5 ± 1.1</td>
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<tr>
<td>D7</td>
<td>4.9 ± 0.7</td>
<td>26.2 ± 5.1</td>
<td>3.2 ± 0.1</td>
<td>22.3 ± 3.8</td>
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Antiserum against glial fibrillary acidic protein, a specific marker of astrocytes (32). Fig. 5 shows some representative results. Staining with the antiserum clearly indicated expression of this marker protein in the cloned cells (Fig. 5, A, C, and D) and C-6 cells (37), a glial cell line derived from rat astrocytoma (Fig. 5E) whereas staining with a non-immune serum gave few positive cells (Fig. 5, B and F). All other clones established here expressed this marker protein (data not shown). Epo production by C-6 cells was undetectable in either 21 or 1% O2.

DISCUSSION

In fetal stage, Epo is produced in the liver where erythropoiesis takes place. In adult, Epo produced by the kidney travels in the circulation to reach erythropoietic tissues, bone marrow, and spleen (in rat, mouse, etc.). So far the erythroid lineage has been thought to be a sole physiological target of Epo. The present study provides a new site for Epo production by demonstrating that astrocytes produce the bioactive Epo. Taken together with the previous finding that neurons express the functional Epo-R (19, 20), our present results support the idea that Epo exerts a novel function in brain.

A much higher concentration of Epo is required to show activity in neurons than in erythroid precursor cells. In neuronal
cells, Epo acts at a nanomolar range (19, 20) whereas Epo at 100 μM shows maximum activity in erythroid cells (1). Indeed, the ligand affinity of Epo-R in neuronal cells (kD = 10 – 16 nm) is much lower than that in erythroid cells (kD = 95 μM) (19). It has been proposed that neuronal and other cells express an accessory protein that alters the ligand affinity through interaction with Epo-R (19, 38, 39). Glial cells including astrocytes are located very close to or interact directly with neurons. Production of Epo by astrocytes indicates that Epo acts on neurons through a paracrine mechanism that would overcome the low ligand affinity of Epo-R in neurons and also the unavailability of blood Epo in the central nervous system due to the blood-brain barrier.

Epo has been shown to elevate monoamine contents and induce rapid incorporation of Ca2+ in the rat cell line PC12, which expresses more neuronal properties with exposure to nerve growth factor (19). Furthermore Epo augments choline acetyltransferase activity in mouse embryonic primary septal neurons and in cholinergic cell line SN6 and promotes the survival of septal cholinergic neurons in adult rats that received fimbria-fornix transections (20). It appears from these results that Epo functions as a neurotrophic factor. Immunochemical experiments with mouse postimplantation embryo have indicated that Epo and Epo-R are expressed in the neural plate, implying that Epo is involved in neurogenesis (40). Further studies are necessary to know a true function of Epo in nervous system.

In accordance with a widely accepted notion that oxygen plays a key role in regulating Epo expression (1, 2, 41), Epo production by astrocytes was dependent on oxygen tension for culture, and the regulation operated at the level of mRNA. No renal cell lines producing Epo in an oxygen-regulated way have been found. Hepatoma cell lines producing Epo are extensively used for in vitro studies of the expression of Epo gene (16, 17). Analyses with reporter gene revealed that the 3′-flanking region of the Epo gene contained an enhancer element responsible for hypoxia-induced transcriptional activation (42-46). The 5′-promoter region is also involved in the hypoxia inducibility (45, 47, 48). Proteins binding to the 3′- and 5′-flanking regions have been reported (42, 49-53). These proteins are also hypoxia-inducible and may be trans-activators in the Epo gene transcription. Astrocyte cell lines established in the present study would be very useful for identification of the trans-acting proteins that regulate expression of the Epo gene in brain.

REFERENCES

Brain Erythropoietin