

# Erythropoietin Gene Expression in Human, Monkey and Murine Brain

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## Abstract

The haematopoietic growth factor erythropoietin is the primary regulator of mammalian erythropoiesis and is produced by the kidney and the liver in an oxygen-dependent manner. We and others have recently demonstrated erythropoietin gene expression in the rodent brain. In this work, we show that cerebral erythropoietin gene expression is not restricted to rodents but occurs also in the primate brain. Erythropoietin mRNA was detected in biopsies from the human hippocampus, amygdala and temporal cortex and in various brain areas of the monkey *Macaca mulatta*. Exposure to a low level of oxygen led to elevated erythropoietin mRNA levels in the monkey brain, as did anaemia in the mouse brain. In addition, erythropoietin receptor mRNA was detected in all brain biopsies tested from man, monkey and mouse. Analysis of primary cerebral cells isolated from newborn mice revealed that astrocytes, but not microglia cells, expressed erythropoietin. When incubated at 1% oxygen, astrocytes showed >100-fold time-dependent erythropoietin mRNA accumulation, as measured with the quantitative reverse transcription–polymerase chain reaction. The specificity of hypoxic gene induction in these cells was confirmed by quantitative Northern blot analysis showing hypoxic up-regulation of mRNA encoding the vascular endothelial growth factor, but not of other genes. These findings demonstrate that erythropoietin and its receptor are expressed in the brain of primates as they are in rodents, and that, at least in mice, primary astrocytes are a source of cerebral erythropoietin expression which can be up-regulated by reduced oxygenation.

## Introduction

The haematopoietic growth factor erythropoietin regulates erythroid differentiation in the haematopoietic organs, including the bone marrow, spleen and fetal liver, by preventing apoptosis of erythroid progenitor cells (for reviews see Krantz, 1991; Jelkmann, 1992; Koury and Bondurant, 1992). When individuals are subjected to hypoxic conditions, formation of erythropoietin is governed by an operationally defined oxygen sensor that leads to the accumulation of mRNA, resulting in elevated erythropoietin serum levels (Krantz, 1991; Jelkmann, 1992). However, the mechanisms by which cells perceive changes in oxygen concentration remain to be elucidated. The localization of erythropoietin synthesis was originally deduced from organ extirpation experiments and more recently from the analysis of erythropoietin mRNA distribution, to reveal that the kidney and liver are the dominant organs of erythropoietin production (for review see Jelkmann, 1992). In the kidney, peritubular interstitial fibroblasts have been identified as erythropoietin-producing cells (Koury *et al.*, 1988), whereas in the liver both hepatocytes and the non-parenchymal Ito cells have been found to express erythropoietin (Koury *et al.*, 1991; Maxwell *et al.*, 1994).

Induced erythropoietin gene expression in cell culture has been

observed after exposing the human hepatoma cell lines HepG2 and Hep3B to either 1% oxygen or cobalt chloride (Goldberg *et al.*, 1987). Molecular analysis using these cell lines as well as transgenic mice carrying the human erythropoietin transgene (Semenza *et al.*, 1991) has revealed the presence of an oxygen-dependent transcriptional enhancer lying 3' to the poly(A) addition site of the erythropoietin gene (Pugh *et al.*, 1991). The nuclear hypoxia-inducible factor 1 (HIF-1), a basic helix–loop–helix heterodimeric transcription factor (Wang *et al.*, 1995), was found to bind to this enhancer sequence in response to reduced oxygen concentrations (Semenza and Wang, 1992). Significantly, oxygen-dependent HIF-1 expression was observed not only in erythropoietin-producing HepG2 and Hep3B cells but also in various other cell lines that do not produce erythropoietin (Wang and Semenza, 1993). In addition, an oxygen-sensing mechanism similar to that regulating gene expression of erythropoietin has also been suggested for the gene encoding the vascular endothelial growth factor (VEGF) in Hep3B cells (Goldberg and Schneider, 1994; Levy *et al.*, 1995), as well as for glycolytic enzymes such as phosphoglycerate kinase 1, aldolase A, pyruvate kinase M and lactate dehydrogenase A (Firth *et al.*, 1994). These

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findings demonstrate that the ability to sense low oxygen concentrations, which leads to up-regulated expression of certain genes, is widespread in mammalian cells (Maxwell *et al.*, 1993).

So far, erythroid precursor cells have been considered to be the main target for erythropoietin. However, recent reports suggest that, beside its role in erythropoiesis, erythropoietin might exert a function in the nervous system; erythropoietin production has been observed in tumours of the human central nervous system, such as cerebellar haemangioblastoma (Trimble *et al.*, 1991) and meningioma (Bruneval *et al.*, 1993). In addition, erythropoietin has been shown to support the survival of lesioned neurons in rats *in vivo* and to augment choline acetyltransferase activity in primary cultured neurons (Konishi *et al.*, 1993). Detection of erythropoietin mRNA in the rat brain (Tan *et al.*, 1992) and our localization of specific erythropoietin binding sites in defined areas of the mouse brain (Digicaylioglu *et al.*, 1995) also support the notion that erythropoietin and its receptor are involved in so far unknown neuronal processes. Erythropoietin gene expression in the brain, in contrast to the kidney and liver, implies that this growth factor acts locally, since transport of cerebrally produced erythropoietin to the blood is most probably impeded by the blood-brain barrier (for review see Bradbury, 1993). By analogy to the situation in the kidney and liver, where erythropoietin gene expression is well established in all mammals, including primates, one would expect that cerebral erythropoietin gene expression is not restricted to mice and rats but also occurs in other species. To test this hypothesis, we analysed human, monkey (*Macaca mulatta*) and mouse brain biopsies for the presence of erythropoietin and erythropoietin receptor transcripts by means of quantitative reverse transcription-polymerase chain reaction (RT-PCR), and established primary cell cultures from the mouse brain to identify possible candidate cells for the production of erythropoietin in this organ. Furthermore, we investigated the ability of the brain to sense and respond specifically to a reduced oxygen supply.

## Materials and methods

### Human brain biopsies

Human brain material was obtained from two women and four men, aged 19–31 years, who had undergone trans-sylvian selective amygdalohippo-parahippocampectomy (Yasargil *et al.*, 1993) for the treatment of intractable temporal lobe epilepsy. The experimental protocol was approved by the University ethical committee. Biopsies from the temporal cortex, amygdala and hippocampus were frozen in liquid nitrogen.

### Animals

Animals were handled in accordance with a protocol approved by the Kantonales Veterinäramt Zürich. Adult male monkeys (*M. mulatta*) used for neuroanatomical studies were subjected to hypoxic conditions prior to perfusion with fixatives. Anaesthetized (40 mg pentobarbital/kg body wt i.p.) and intubated animals breathed a gas mixture containing either 8 or 20% oxygen for 2 h. Arterial blood gases were analysed during ventilation using a blood gas analyser (IL 1304, Instrumentation Laboratory, Milan, Italy). Tissue samples from the kidney and liver as well as from various areas of the brain (temporal, frontal and occipital cortex, cerebellum, hypothalamus, hippocampus and nucleus caudatus) were taken and transferred to liquid nitrogen.

Stimulation of erythropoietin production in adult male C57BL/6 mice was achieved by carbon monoxide inhalation resulting in functional anaemia (Eckardt *et al.*, 1992). Animals were exposed for 4 h in an airtight chamber that was supplied with a mixture of normal

air supplemented with 0.1% carbon monoxide. Immediately after exposure, mice were killed by decapitation, and after collecting blood, the kidneys, liver, brain, testis, spleen and samples of skeletal muscle were removed and frozen in liquid nitrogen. Hypoxic stimulation was monitored by determination of serum erythropoietin concentration using a radioimmunoassay as described (Eckardt *et al.*, 1988).

### Primary brain cell culture

Primary glial cell cultures were prepared from brains of newborn C57BL/6 mice as described (Frei *et al.*, 1986, 1987). Following removal of the meninges, the brains were mechanically dissociated by pressing through nylon sieves. The cells were seeded in DME medium (Gibco-BRL) containing 20% fetal calf serum (FCS; Boehringer Mannheim), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 × Minimal Essential Medium non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate (all from Gibco-BRL) in 175 cm<sup>2</sup> tissue culture flasks (two brains per flask), and were cultured in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Fresh medium with reduced FCS concentrations was added to the cells at days 6 (10% FCS) and 9 (5% FCS) of culture. Confluent cultures at day 13 were used to harvest both microglia cells and astrocytes. After agitating the confluent culture (500 r.p.m.) for 1 h, the adherent cells were saved for astrocyte preparation (see below), and the floating cells were transferred to a fresh tube to isolate microglia cells. After 10 min of gravity sedimentation at room temperature, the sediment was discarded and the supernatant containing floating cells was incubated at 37°C for 2 h in a flask and then gently agitated (70 r.p.m.) for an additional 10 min. Subsequently, the non-adherent cells present in the medium were removed while the remaining adherent cells were identified as microglia cells by immunofluorescence. The isolation of astrocytes followed the same protocol until day 13, when the floating microglia cells were removed (see above). The adherent cells were trypsinized and subcultured in medium containing 5% FCS. After 3 additional days, >95% of these first-passage cells were identified as astrocytes by immunofluorescence. Exposure to a low oxygen concentration was performed with confluent astrocytes: after replacing the medium (5% FCS), the cells were incubated for 6–94 h at either 20% O<sub>2</sub> (140 mm Hg, normoxia) or 1% O<sub>2</sub> (7 mm Hg, hypoxia) with or without addition of 100 µM CoCl<sub>2</sub> or 15 ng/ml human recombinant interleukin-6 (IL-6; R&D Systems, Basel, Switzerland) in a Forma Scientific incubator (Model 3319, Brouwer, Luzern, Switzerland).

### Immunofluorescent staining for glial fibrillary acidic protein and Fc receptor

For immunofluorescent staining, antibodies were diluted in phosphate-buffered saline containing 0.1% bovine serum albumin. Whereas astrocytes were directly cultured on glass slides, the microglia cells were cytopun at a density of 1000 cells per slide. For staining of the astrocyte-specific intermediate filaments, slides were incubated with a 1:400 dilution of a rat anti-glial fibrillary acidic protein (GFAP) antiserum (Sigma) for 30 min at 25°C. To examine the Fc (IgG<sub>1/2b</sub>) receptor, a marker for microglia cells (Frei *et al.*, 1987), cells were incubated with monoclonal rat anti-mouse Fc receptor antibody (New England Nuclear) using a 1:1000 dilution for 30 min at 25°C. Subsequently, the cells were washed three times in PBS and incubated for 30 min with a 1:100 dilution of a biotinylated anti-rat IgG antibody (Sigma). As negative controls, cells were incubated with the second antibody alone. After washing, fluorescein isothiocyanate-conjugated streptavidin (Sigma) at 1:100 dilution was incubated for 30 min and the slides were examined in a Zeiss laser scanning fluorescence microscope.

### RNA preparation and Northern blot analysis

Frozen tissue was weighed and total RNA was isolated according to Chomczynski and Sacchi (1987). Resuspended RNA was quantified by spectrophotometric absorbance at 260 nm and tested for integrity by 1% agarose gel electrophoresis containing 0.05% sodium dodecyl sulphate in the running buffer. Total RNA (10 µg) was denatured in formamide/formaldehyde and electrophoresed through a 1% agarose gel containing 6% formaldehyde as described (Sambrook *et al.*, 1989). After pressure blotting (Stratagene) to nylon membranes (Biodyne A, Pall, Winiger, Wohlen, Switzerland), the RNA was hybridized to <sup>32</sup>P-labelled probes following the manufacturer's (Pall) instructions. The radioactive signal was recorded and quantified using a phosphorimager (Molecular Dynamics, Paul Bucher, Basel, Switzerland). The mouse VEGF probe was derived from a 644 bp PCR fragment generated by using primers 5'-GCGGGCTGCCTC-GCAGTC-3' and 5'-TCACCGCCTTGGCTTGTCAC-3' respectively (corresponding to bp 16–33 and 659–640 of the mouse VEGF cDNA sequence; numbering according to Claffey *et al.*, 1992). The contrapsin probe was a kind gift from Dr Y. Suzuki (Osaka, Japan). Complement C3 cDNA was purchased from ATCC (phACT235, ATCC No. 61600, Rockville, MD) and the human actin probe was obtained from Clontech.

### Construction of competitor erythropoietin cRNA templates

The human competitor erythropoietin template was constructed by deleting a 102 bp *PvuII*–*PvuII* erythropoietin cDNA fragment (corresponding to bp 2405–2642 of the genomic erythropoietin sequence; numbering according to Jacobs *et al.*, 1985) from the human erythropoietin cDNA-containing plasmid pE49f. The *KpnI*–*SacI* erythropoietin cDNA fragment (1307–3052), harbouring the 102 bp deletion, was subcloned into vector pSP64polyA (Promega, Catalys, Wallisellen, Switzerland), giving rise to plasmid pEPO64. After cleavage with *EcoRI* and gel purification, the linearized plasmid was transcribed *in vitro* using SP6 RNA polymerase (Fermentas, Mächler, Basel, Switzerland) according to the manufacturer's directions, and the synthesized cRNA was quantified by absorbance at 260 nm. The resulting competitor product, following RT–PCR, was 178 bp long compared to 280 bp of the amplified target erythropoietin mRNA using the same primer set, allowing easy separation of both fragments by agarose gel electrophoresis. The mouse competitor erythropoietin template was obtained by deleting the *ScaI*–*SmaI* fragment (corresponding to bp 1568–2980; numbering according to Shoemaker and Mitsok, 1986) located in exon 2 and 5 of the mouse genomic erythropoietin fragment *BamHI*–*XmnI* (856–3463 bp) subcloned into pSP64polyA (*BamHI*–*SmaI*), giving rise to pLR1-EPO. The mouse competitor erythropoietin cRNA was obtained as described above and the RT–PCR product was 492 bp long, compared to 395 bp of the amplified target erythropoietin mRNA.

### Quantitation of erythropoietin mRNA by competitive RT–PCR

Both competitor and sample RNA species were reverse-transcribed into cDNA using Promega's Reverse Transcription System. To melt secondary structures, the RNA was denatured at 60°C for 10 min and chilled on ice. Subsequently, a series of 20 µl reaction mixtures containing 1 µg total RNA, each spiked with 0.2 fg to 20 pg of synthetic erythropoietin cRNA, 1 × reverse transcription buffer [10 mM Tris–HCl (pH 8.8 at 25°C), 50 mM KCl and 0.1% Triton X-100], 1 mM each dNTP's, 5 mM MgCl<sub>2</sub>, 20 U rRNasin, 15 U avian myeloblastosis virus reverse transcriptase and 0.5 µg oligo(dT)<sub>15</sub> primer were incubated for 1 h at 42°C. The reaction was terminated by heating the samples for 5 min at 75°C. Two negative controls

were carried out for each RT–PCR set by omitting either avian myeloblastosis virus reverse transcriptase or RNA from the reaction mixture.

PCR for human and monkey erythropoietin was performed with the sense primer located in exon 3 (5'-CGTCCCAGACACCAAAGTT-3') and the antisense primer located in exon 5 (5'-AGTGATGGTT-CGGAGTGGA-3') corresponding to bp 1639–1657 and 2666–2648 respectively (numbering according to Jacobs *et al.*, 1985) in a reaction mixture containing 1 × polymerization-buffer [10 mM Tris–HCl (pH 9.0 at 25°C), 50 mM KCl, 0.01% (wt/vol) gelatin, 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>], 0.2 mM each dNTP's, 25 pmol of each primer, 0.2 U Super Taq Polymerase (Staehelin, Basel, Switzerland) and 5 µl from each reverse transcription reaction in a final volume of 50 µl. After initial denaturation for 1 min at 94°C, Taq polymerase was added and PCR was performed for 30–35 cycles (GeneAmp PCR System 9600 thermocycler, Perkin Elmer Cetus) with denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, elongation at 72°C for 1.5 min, and final elongation at 72°C for 5 min. PCR for mouse erythropoietin was performed as described (Digicaylioglu *et al.*, 1995).

Following agarose gel electrophoresis, the relative amount of target cDNA versus competitor DNA was quantitated by pixel analysis of video camera recordings. Coaccumulation of the two cDNA species was verified by erythropoietin RT–PCR analysis using either the competitor cRNA template (250 fg/assay) or 0.125 µg total RNA from kidney of stimulated mice containing ~250 fg erythropoietin mRNA/µg total RNA. The reactions were stopped after 25, 27, 29, 31 and 33 cycles and the amplified products were separated by gel electrophoresis. Analysis of the ethidium bromide-stained gel revealed a similar increase in both products after the same number of amplification cycles. Reproducibility of erythropoietin mRNA quantitation by competitive RT–PCR was verified by quadruplicate measurements of dilution series from the same sample, resulting in essentially identical values.

### Detection of erythropoietin receptor mRNA by RT–PCR

Reverse transcription of total RNA samples was performed as described above. An aliquot (3 µl) from this reaction was used as a template for amplification (30–35 cycles). For primate erythropoietin receptor, RT–PCR was performed using the sense primer located in exon 3 (5'-ACCGTGTCATCCACATCAAT-3') and the antisense primer in exon 7 (5'-GCCTTCAAACCTCGCTCTCTG-3') (corresponding to bp 401–420 and 885–866 respectively of the human erythropoietin receptor cDNA sequence; numbering according to Jones *et al.*, 1990). The amplification profile was 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. The resulting erythropoietin receptor product was 485 bp long. One hundred femtograms of human erythropoietin receptor cDNA was used as a positive control. Murine erythropoietin receptor mRNA was detected as described (Digicaylioglu *et al.*, 1995) using total RNA from murine erythro-leukaemia (MEL) cells as positive control (D'Andrea *et al.*, 1989).

### Presentation of data and statistics

Where appropriate, data are given as mean ± SD of *n* experiments. Student's *t*-test was used to evaluate the significance of differences.

## Results

### Hypoxic induction of erythropoietin mRNA in the primate brain

Human brain biopsies were used to test whether erythropoietin is transcribed in this organ as it is in the rodent brain. To this end, we

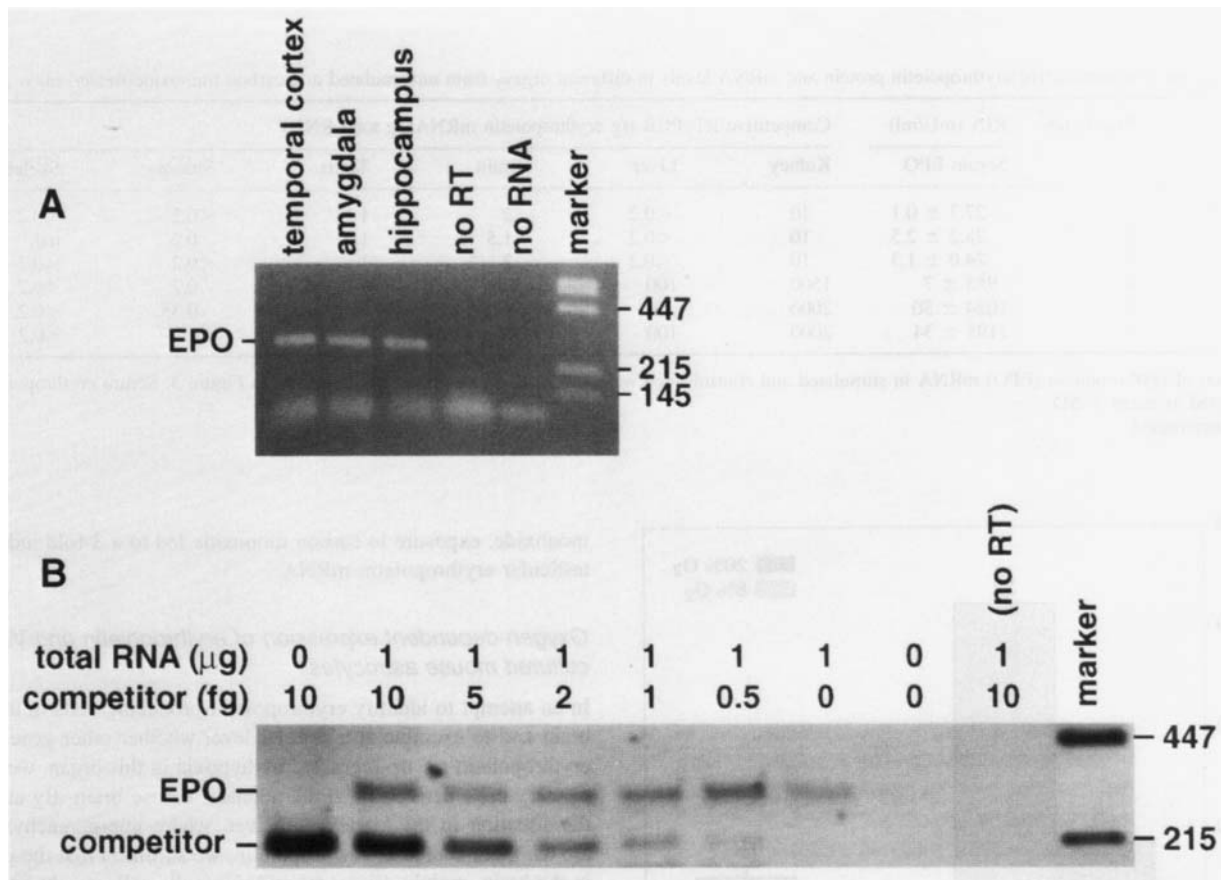


FIG. 1. Detection and quantitation of erythropoietin (EPO) transcripts in human brain tissues by RT-PCR. One microgram each of total RNA from human temporal cortex, amygdala and hippocampus (A) or 1 µg of total RNA from hippocampus spiked with the indicated amount of competitor erythropoietin cRNA (B) was reverse-transcribed and amplified. After gel electrophoresis in 1.4% agarose, the reaction products were stained with ethidium bromide and photographed (A) or quantitated by pixel analysis of a video camera recording (B). The RT-PCR product length for primate erythropoietin is 280 bp and for the competitor 178 bp. No RT, no reverse transcriptase added.

took advantage of the therapeutic treatment of six epileptic patients who had undergone unilateral amygdala-hippocampectomy. Total RNA samples isolated from these biopsies were analysed by RT-PCR, revealing that erythropoietin mRNA was present in all three brain areas, as exemplified in Figure 1A. The identity of the amplified 280 bp erythropoietin product was further confirmed by restriction fragment length analysis (not shown). To quantify erythropoietin mRNA levels in the primate brain, we established a competitive RT-PCR assay. This technique involves reverse transcription of the test samples spiked with varying amounts of a synthetic erythropoietin cRNA added as standard, and coamplification of the cDNA products by PCR using primers common to both templates, thus correcting for variability in the efficiency of reverse transcription and amplification. A representative quantitation experiment from a hippocampus biopsy is shown in Figure 1B. Independent measurements revealed that  $1.5 \pm 0.5$  fg erythropoietin mRNA per µg total RNA ( $n = 3$ ) was present in human hippocampus as well as in the biopsies of temporal cortex and amygdala.

Because it was not possible to investigate erythropoietin mRNA regulation in the brain of humans after oxygen deprivation, we used monkeys to test whether erythropoietin mRNA is up-regulated in the brain of hypoxic primates. Each two anaesthetized monkeys spontaneously breathed a gas mixture containing either 20% (normoxia) or 8% oxygen (hypoxia), the latter mimicking the oxygen concentration found at an altitude of 7500 m above sea level. Blood

gas analysis revealed that after 2 h exposure to 8% oxygen, the arterial  $pO_2$  was 44 mm Hg (5.9 kPa) and the oxygen saturation was diminished to 58.5%, compared to 75 mm Hg (10.0 kPa) and 92.0% respectively in the normoxic control animals. Furthermore, erythropoietin serum levels were found to be elevated by a factor of 2.5 as measured by radioimmunoassay. These findings confirmed that the exposed animals were indeed hypoxic. Competitive RT-PCR analysis revealed that erythropoietin mRNA levels increased more than 200 times ( $P < 0.01$ ) in the hypoxic kidney, reaching a concentration of  $1667 \pm 416$  fg erythropoietin mRNA/µg total RNA (Fig. 2). Similar to human biopsies (see above), normoxic monkey brain biopsies contained  $1.16 \pm 0.4$  fg erythropoietin mRNA/µg total RNA, independently of the sample's origin (e.g. temporal, frontal or occipital cortex, cerebellum, hypothalamus, hippocampus or nucleus caudatus). Hypoxic exposure led to an increase of ~3-fold ( $P < 0.001$ ) in the erythropoietin mRNA concentration in brain tissue (Fig. 2).

#### Quantitation of erythropoietin mRNA in hypoxic mouse organs

Exposure of animals to 0.1% carbon monoxide results in functional anaemia and represents a strong stimulus for erythropoietin expression (Eckardt *et al.*, 1992). Since inhalation of carbon monoxide by the monkeys was not practicable, we exposed mice to 0.1% carbon monoxide for 4 h (see Materials and methods). Blood sample analysis revealed that ~50% of the haemoglobin was bound to carbon monoxide

TABLE 1. Serum immunoreactive erythropoietin protein and mRNA levels in different organs from unstimulated and carbon monoxide-treated mice

	Mouse no.	RIA (mU/ml)	Competitive RT-PCR (fg erythropoietin mRNA/ $\mu$ g total RNA)					
		Serum EPO	Kidney	Liver	Brain	Testis	Spleen	Skeletal muscle
Unstimulated (20% O <sub>2</sub> )	1	27.3 $\pm$ 0.1	10	<0.2	2	1	<0.2	<0.2
	2	28.2 $\pm$ 2.5	10	<0.2	1.5	1.5	0.2	n.d.
	3	24.0 $\pm$ 1.3	10	<0.2	2	1	<0.2	<0.2
Stimulated (0.1% CO, 4 h)	4	953 $\pm$ 7	1500	100	30	4	0.2	<0.2
	5	1024 $\pm$ 30	2000	50	30	2	0.35	<0.2
	6	1195 $\pm$ 34	2000	100	30	4	n.d.	<0.2

Quantitation of erythropoietin (EPO) mRNA in stimulated and control mice was performed as described in the legend to Figure 3. Serum erythropoietin levels are expressed as mean  $\pm$  SD.

n.d., not determined.

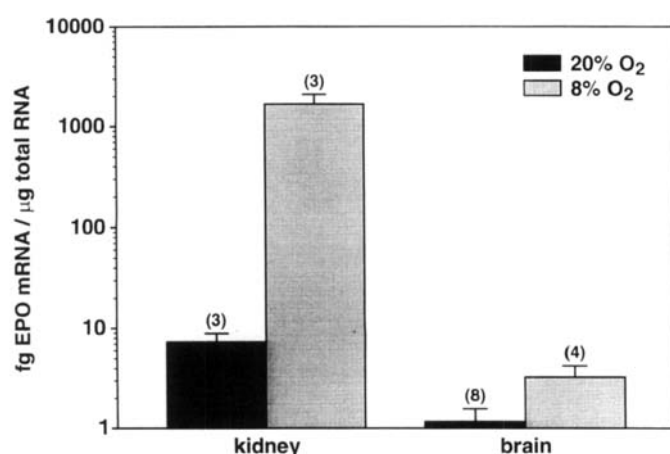


FIG. 2. Quantitation of erythropoietin (EPO) mRNA in normoxic and hypoxic monkeys. Total RNA was isolated from normoxic and hypoxic monkeys previously breathing 20 and 8% O<sub>2</sub> respectively for 2 h, and the erythropoietin mRNA level was quantitated by competitive RT-PCR. Biopsies were taken from the kidney (positive control) and different areas of the brain. The difference between normoxic and hypoxic organs was significant according to Student's *t*-test (kidney,  $P < 0.01$ ; brain,  $P < 0.001$ ,  $n = 3-8$  as indicated).

(as measured by the IL-CO-Oximeter 282, Milan, Italy). Hypoxic stimulation of erythropoietin production was confirmed by a 40-fold increase in serum erythropoietin protein level (as shown in Table 1). Quantitation of mouse erythropoietin mRNA in several organs was performed by competitive RT-PCR, as exemplified by mouse no. 1 (normoxia) and no. 6 (carbon monoxide-exposed) in Figure 3. Table 1 summarizes the values measured in six animals. In the kidney, the main erythropoietin-producing organ, induction was nearly 200-fold; erythropoietin mRNA levels increased from 10 fg/ $\mu$ g total RNA in control mice ( $n = 3$ ) to  $1833 \pm 289$  fg/ $\mu$ g total RNA in carbon monoxide-treated animals ( $n = 3$ ). Hepatic erythropoietin mRNA rose from barely detectable levels to 100 fg/ $\mu$ g total RNA. In the brain, we observed an increase in erythropoietin mRNA levels from  $1.83 \pm 0.29$  fg erythropoietin mRNA/ $\mu$ g total RNA in control mice to 30 fg/ $\mu$ g total RNA in carbon monoxide-treated animals, representing an induction factor of  $\sim 20$  (see also Fig. 3). Low level erythropoietin mRNA was also found in testis ( $1.17 \pm 0.29$  fg/ $\mu$ g total RNA) as well as in the spleen of control mice. Interestingly, while no erythropoietin transcripts were detected in skeletal muscle, independently of whether or not the mice were exposed to carbon

monoxide, exposure to carbon monoxide led to a 3-fold induction of testicular erythropoietin mRNA.

#### Oxygen-dependent expression of erythropoietin and VEGF in cultured mouse astrocytes

In an attempt to identify erythropoietin-producing cells in the mouse brain and to examine at a cellular level whether other genes besides erythropoietin are up-regulated by hypoxia in this organ, we cultured primary cells derived from the neonatal mouse brain. By analogy to the situation in the kidney and liver, where non-parenchymal cells are the main source of erythropoietin, we surmised that the glial cells in the brain, mainly astrocytes and microglia cells, might be a source of erythropoietin production. Thus, we established cultures enriched in microglia cells and astrocytes. The identity of the two cell types was verified using antibodies raised against the Fc receptor (IgG<sub>1/2b</sub>) (microglia cells) and GFAP (astrocytes). Immunofluorescence analysis revealed that  $>90\%$  of the cells in the microglia fraction were positive for the Fc receptor whereas GFAP-positive cells represented  $<5\%$  of all cells (Fig. 4). On the other hand,  $>95\%$  of the cells present in the astroglia fraction were GFAP-positive (Fig. 4), thus showing efficient separation of the two cell types.

Analysis of total RNA from normoxic cells revealed the presence of erythropoietin mRNA in primary astrocytes only. Quantitative RT-PCR revealed that the erythropoietin mRNA level in these cells was up-regulated more than 100-fold by hypoxia (1% oxygen) in a time-dependent manner (Fig. 5). A very faint erythropoietin RT-PCR signal was found in cultured primary microglia and oligodendrocytes only after hypoxic induction (data not shown), most probably resulting from contaminating astrocytes. These results identified astrocytes as erythropoietin-producing cells and demonstrated that these cells are capable of increasing the erythropoietin mRNA level in an oxygen-dependent manner. Interestingly, addition of cobalt chloride, which is known to induce erythropoietin expression in human hepatoma cell lines (Goldberg *et al.*, 1987), showed no significant stimulatory effect in astrocytes (Fig. 5).

To verify that hypoxic up-regulation in these cells was specific, we analysed the mRNA levels of other genes after reduced oxygenation. We chose contrapsin and complement C3 because these genes are known to be expressed in astrocytes. We also investigated up-regulation of VEGF mRNA, a potent inducer of angiogenesis (reviewed in Plate *et al.*, 1994), since this gene is expressed in the mammalian brain (Breier *et al.*, 1992) and since hypoxic VEGF up-regulation has been observed in human glioblastomas (Shweiki *et al.*, 1992). In addition, it has been suggested that there exist similarities

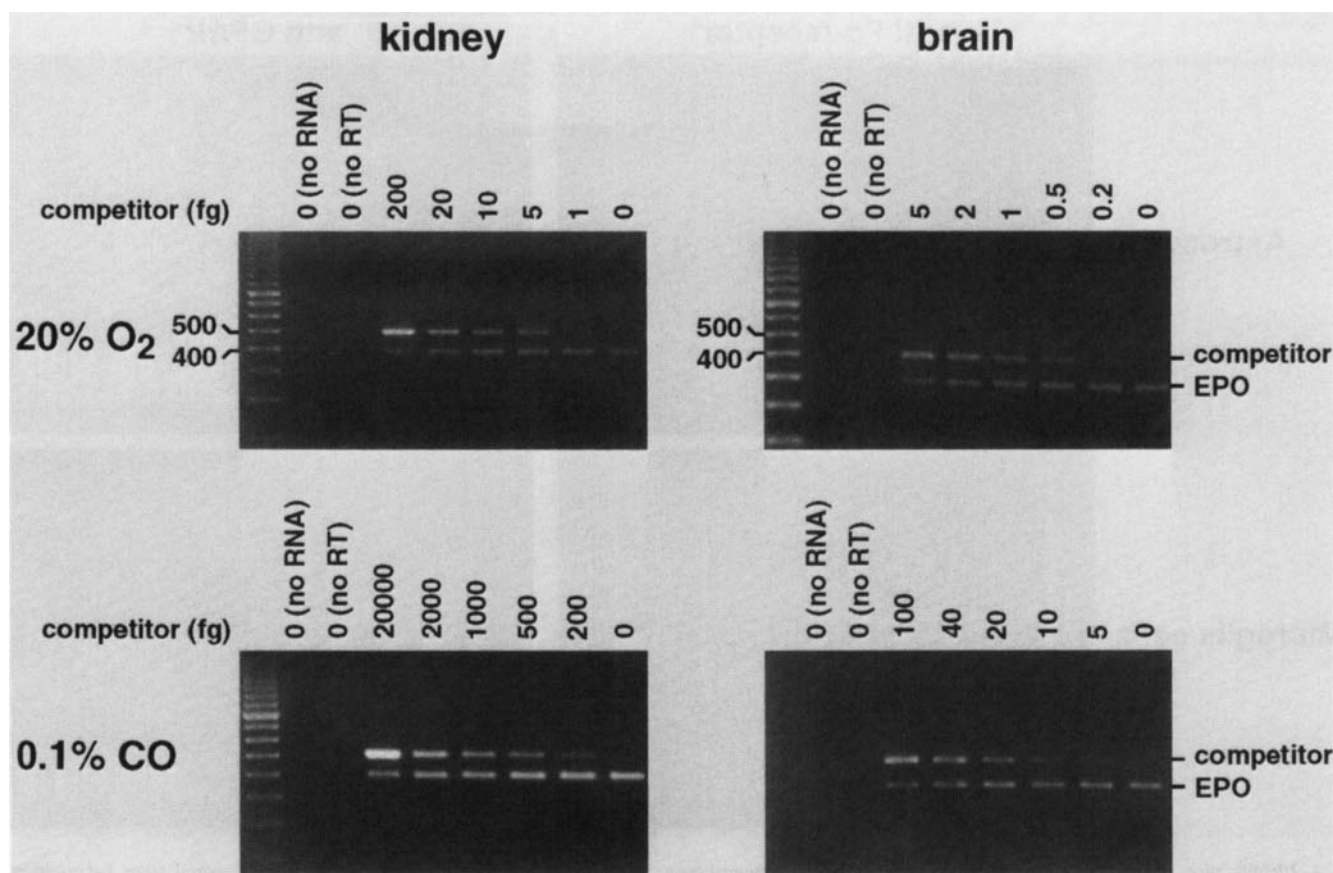


FIG. 3. Quantitation of erythropoietin (EPO) mRNA in stimulated and control mice. Erythropoietin mRNA from kidney and brain of mice exposed to 0.1% CO for 4 h and from unstimulated control animals was quantitated by competitive RT-PCR. The RT-PCR product length for mouse EPO is 395 bp and for the competitor 492 bp. No RT, no reverse transcriptase added; no RNA, no sample RNA added.

between the oxygen-sensing mechanisms regulating the expression of VEGF and erythropoietin (Goldberg and Schneider, 1994; Levy *et al.*, 1995). Northern blot analysis using total RNA isolated from primary astrocytes showed hypoxic induction of VEGF mRNA by up to 9-fold, whereas cobalt chloride led to only a slight increase in VEGF mRNA after 48 h (Fig. 6). In contrast, complement C3 and contrapsin showed no significant increase in mRNA levels after hypoxic or cobalt chloride stimulation. To further study the expression of these genes we exposed normoxic and hypoxic astrocytes to IL-6, since this cytokine activates acute-phase genes in these cells (Potter, 1992). Furthermore, IL-6 was the only cytokine that showed a synergistic effect on erythropoietin induction in a human hepatoma cell line (Faquin *et al.*, 1992). Indeed, addition of IL-6 showed a 1.5-fold synergistic increase in erythropoietin mRNA levels in combination with hypoxia, whereas IL-6 alone had no effect on normoxic astrocytes (Fig. 5). IL-6 did not stimulate VEGF or complement C3 expression either in normoxic or hypoxic astrocytes, and IL-6 only moderately stimulated contrapsin (Fig. 6). It is known, however, that this gene is only induced by IL-6 in the presence of dexamethasone (Potter, 1992). In conclusion, the mRNA levels of erythropoietin and VEGF in astrocytes were specifically up-regulated by hypoxia, and IL-6 affected only hypoxic erythropoietin stimulation.

#### Detection of erythropoietin receptor mRNA in the brain of man, monkey and mouse

Assuming that erythropoietin plays a role in the mammalian brain and considering that erythropoietin most likely does not cross the

blood-brain barrier, one would postulate that erythropoietin acts locally in this organ. This notion requires the expression of erythropoietin receptor in the brain since so far only one erythropoietin receptor is known and erythropoietin exerts its function by binding to this receptor. We therefore analysed all brain tissue samples for the presence of erythropoietin receptor mRNA by means of RT-PCR. As shown in Figure 7A, erythropoietin receptor transcripts were detected in all brain areas tested from man (hippocampus, amygdala and temporal cortex), monkey (temporal cortex, cerebellum, hypothalamus, hippocampus, and nucleus caudatus), and normoxic and carbon monoxide-treated mice (Fig. 7B). In addition to the bone marrow and brain, mouse erythropoietin receptor mRNA was also found in the kidney, testis and spleen. Only marginal amounts were detected in skeletal muscle and liver. These findings support the notion that interactions between erythropoietin and its receptor might have as yet unknown physiological functions in several non-haematopoietic organs of mammals.

#### Discussion

##### Transcription of erythropoietin and erythropoietin receptor in the mammalian brain

We have shown that erythropoietin mRNA is constitutively expressed in all tested areas of normoxic brain of man (hippocampus, amygdala, temporal cortex), the monkey *M. mulatta* (temporal, frontal and occipital cortex, cerebellum, hypothalamus, hippocampus, nucleus

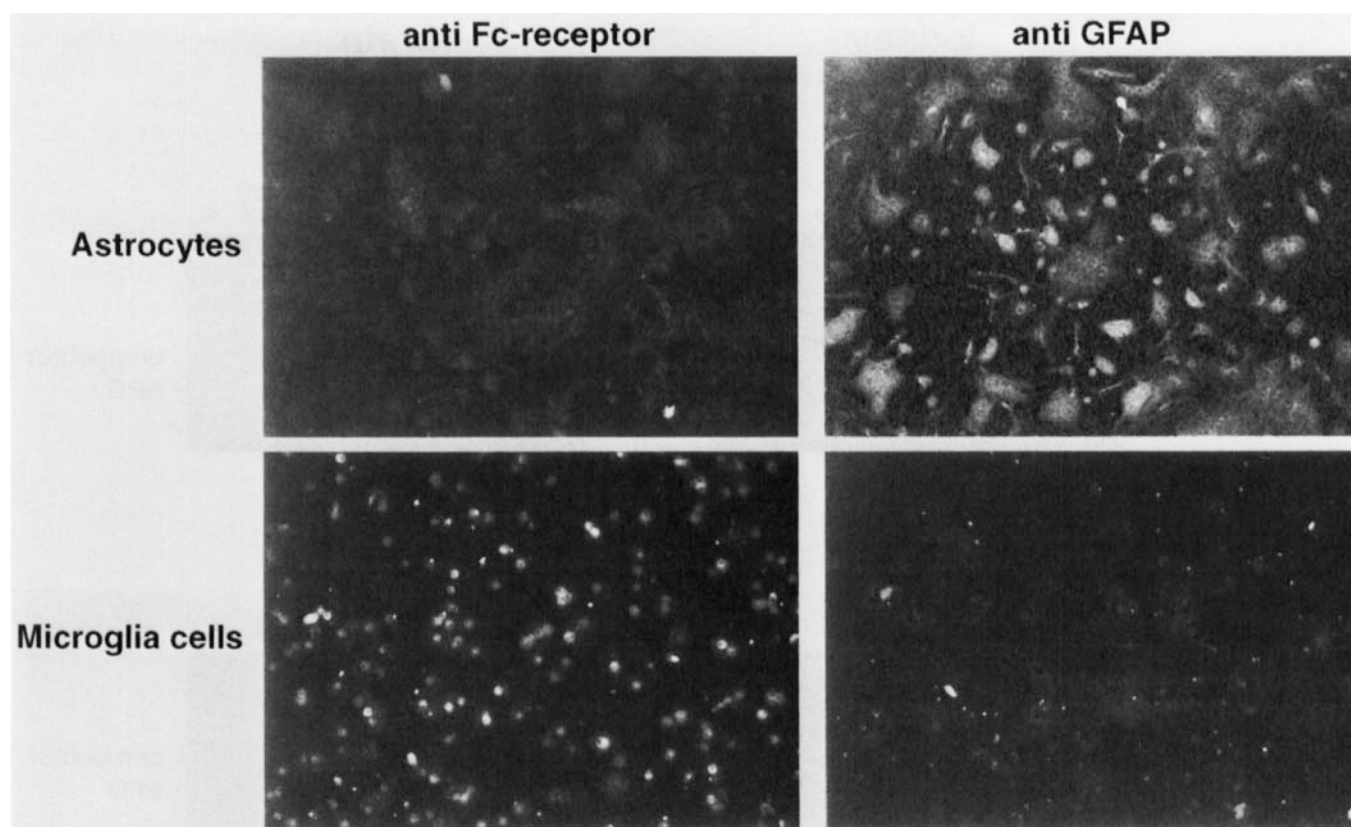


FIG. 4. Identification of astrocytes and microglia cells by immunofluorescence. Primary astrocytes and microglia cells were prepared from 1-day-old mice. Astrocytes and microglia cells were incubated with antibodies raised against the Fc receptor, specific for microglia cells, or GFAP, specific for astrocytes.

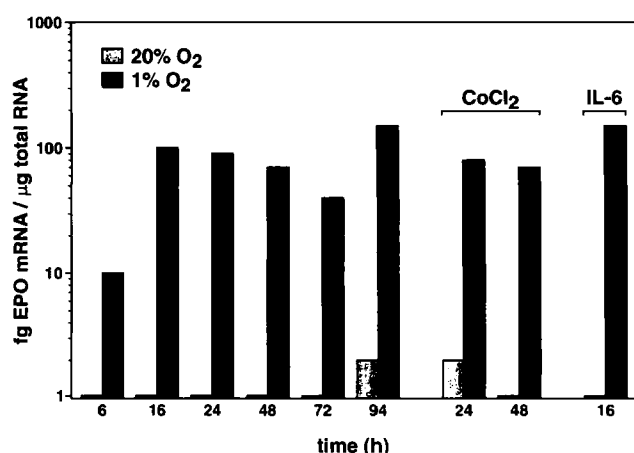


FIG. 5. Quantitation of erythropoietin (EPO) mRNA levels in primary murine astrocytes. Cells were incubated for 6–94 h at 20 or 1% O<sub>2</sub> with or without 100 µM CoCl<sub>2</sub> chloride or 15 ng/ml IL-6. Total RNA was isolated after stimulation of the cells and the erythropoietin mRNA level was determined by competitive RT-PCR.

caudatus) and the mouse. Histological examination of the human brain samples used in this study showed a reactive gliosis in the hippocampus and amygdala (data not shown). This might lead to the speculation that erythropoietin expression is enhanced under these pathological conditions. However, comparable concentrations of erythropoietin mRNA were also found in the normal brain of both monkey and mouse. Together with similar observations in the rodent

brain (Tan *et al.*, 1992; Masuda *et al.*, 1994; Digicaylioglu *et al.*, 1995) our findings support the hypothesis that erythropoietin plays a physiological role in the mammalian brain, including the human brain. This notion is strengthened by the relatively high constitutive erythropoietin mRNA level found in the normoxic mouse brain: when taking organ weight into account and assuming identical transcription efficiencies, accumulation of cerebral erythropoietin mRNA was considerably higher than in the liver and represented ~10% of the constitutive renal erythropoietin production. Furthermore, we showed that erythropoietin gene expression in the brain responds to the physiological stimuli of hypoxia and anaemia (resulting from exposure to carbon monoxide) with an increase in the erythropoietin mRNA level. In comparison to the normoxic control mice, hypoxic up-regulation of erythropoietin mRNA was 10 times higher in the kidney and 2–5 times higher in the liver, compared to the brain. This observation can be explained by assuming that cerebrally produced erythropoietin would remain in the brain as the blood–brain barrier insulates this organ from the bloodstream and most probably hinders renal erythropoietin from entering this tissue. Thus, erythropoietin might act locally as a paracrine rather than as an endocrine factor. As such, much lower amounts of erythropoietin could be sufficient to achieve local concentrations similar to those in the bone marrow, to which erythropoietin has to be transported upon release by the kidney into the bloodstream and subsequent dilution. Alternatively, it is possible that the threshold level of the oxygen sensor is different in the brain compared to the kidney and liver, suggesting that the response to hypoxia is specifically modulated in every organ.

So far, erythropoietin is thought to exert its function exclusively by binding to the erythropoietin receptor. Thus, paracrine action of



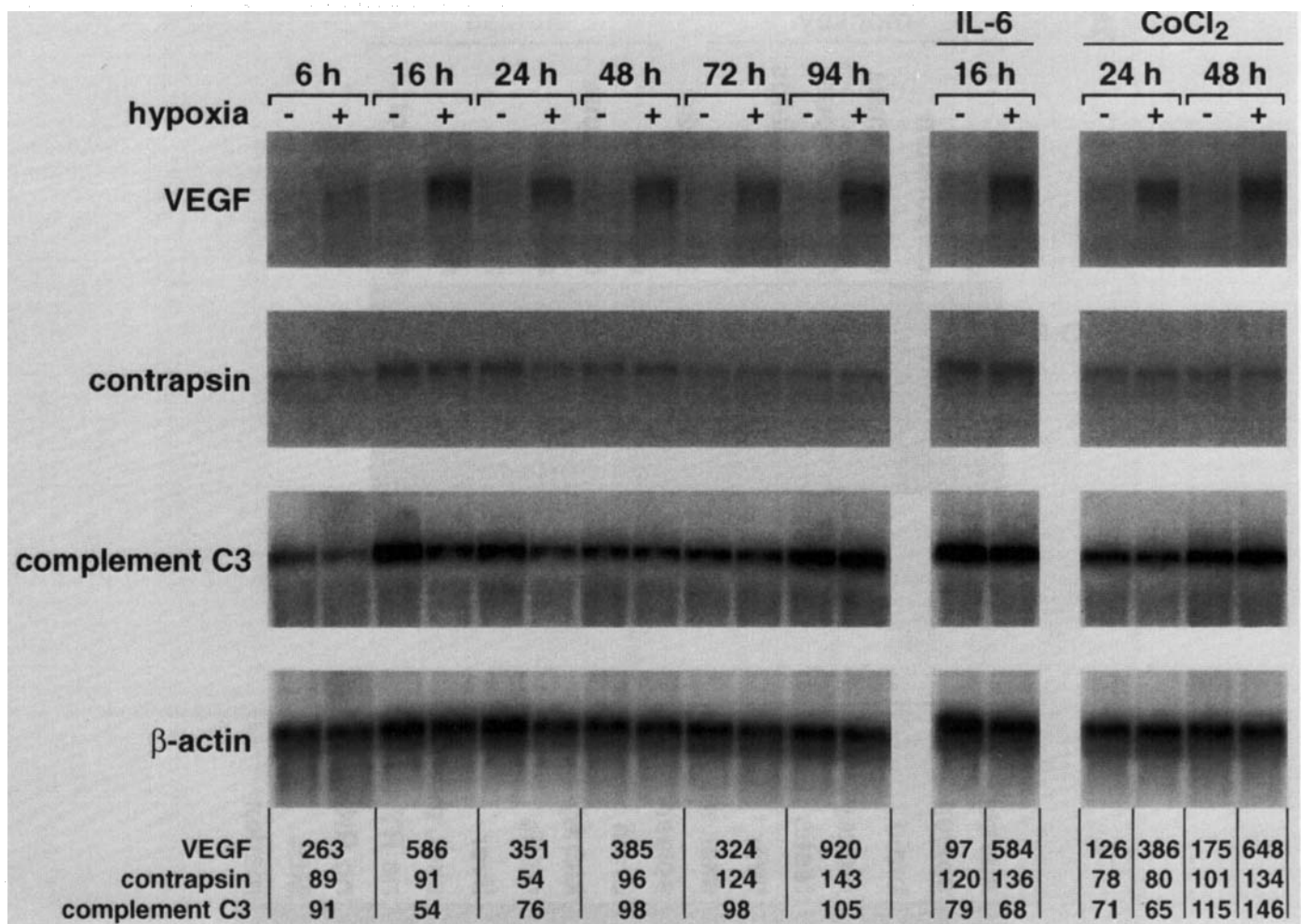


FIG. 6. Quantitative Northern blot analysis of primary astrocytes. Cells were incubated at 20% (–) or 1% O<sub>2</sub> (+) as described in Figure 5. Equal amounts (10 µg) of total RNA were loaded on each lane. After blotting, the membrane was hybridized to probes encoding VEGF, contrapsin, complement C3 and β-actin, the latter serving as a loading, blotting and internal normalization control. The signals were evaluated by phosphorimager analysis, and the values, normalized to β-actin, are shown as percentages of the corresponding normoxic and untreated control value at the bottom of the figure.

erythropoietin in the brain implies the presence of erythropoietin receptor in this organ. We recently reported the presence of specific erythropoietin binding sites and erythropoietin receptor mRNA in the mouse brain (Digicaylioglu *et al.*, 1995). In addition, erythropoietin receptor mRNA has also been observed in the developing mouse brain (Yasuda *et al.*, 1993) and in the non-erythroid clonal cell lines PC12 and SN6, which display neuronal characteristics (Masuda *et al.*, 1993). In this study, we have shown that the erythropoietin receptor gene is expressed in all tested areas of human and monkey brain.

#### Hypoxia-inducible erythropoietin and VEGF gene expression in primary astrocytes

While we detected erythropoietin mRNA in primary cultures of murine astrocytes, only very small amounts were observed in microglia. Hypoxic induction of astrocytes led to a 100-fold increase in the erythropoietin mRNA level. This increase is considerably higher than that found *in vivo* (see above), but can be explained by the much more severe reduction in oxygen concentration used *in vitro*. Our findings are in agreement with a recent report demonstrating that transformed rat astrocytes harbouring the SV40 large T-antigen produced erythropoietin in an oxygen-dependent manner (Masuda

*et al.*, 1994). Interestingly, cobalt chloride, an inducer of erythropoietin production in the hepatoma cell lines HepG2 and Hep3B (Goldberg *et al.*, 1987), had no effect on erythropoietin mRNA levels in primary astrocytes, a phenomenon which has also been reported for primary cultures of rat hepatocytes, which represent the only other primary cells capable of erythropoietin production (Eckardt *et al.*, 1993). Addition of IL-6 to the primary astrocytes synergistically increased hypoxic erythropoietin mRNA levels by 50%, whereas IL-6 alone had no effect under normoxic condition. These findings very closely resemble previous results obtained with the hepatoma cell line Hep3B (Faquin *et al.*, 1992). However, it is unknown how erythropoietin-producing primary hepatocytes respond to IL-6. Unfortunately, the lack of a regulated erythropoietin-producing cell line of renal origin impedes a comparison with astrocytes.

To test whether oxygen-dependent gene expression in primary astrocytes is restricted to erythropoietin, we analysed the mRNA level of VEGF, another hypoxia-inducible gene. We showed that astrocytes are a source of VEGF production in the brain and that stimulation by hypoxia or cobalt chloride led to a significant increase in VEGF mRNA levels, whereas addition of IL-6 had no effect. These results were confirmed by a recent study, published while this



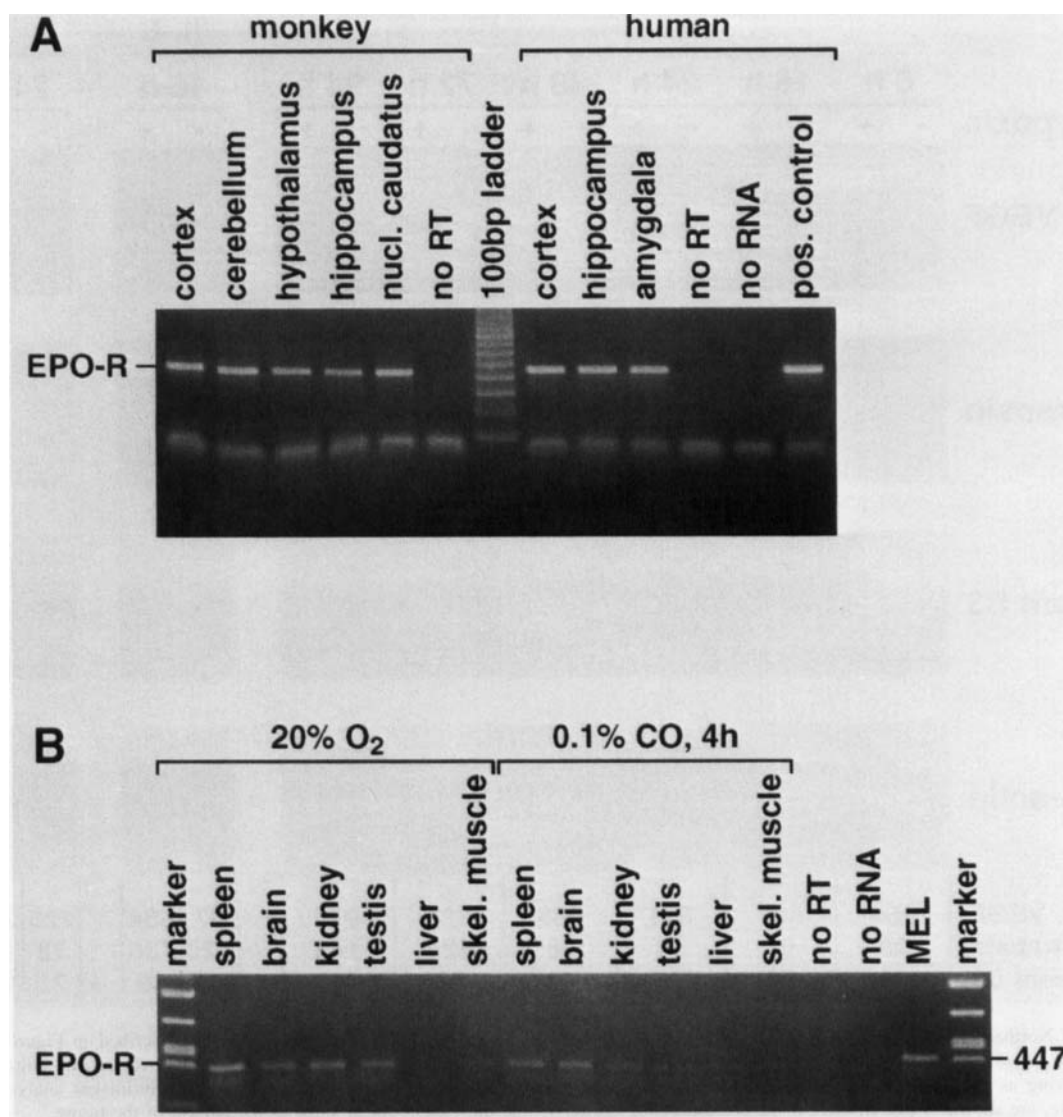


FIG. 7. Detection of erythropoietin receptor (EPO-R) mRNA in different regions of the primate (monkey and human) brain (A) and in mouse organs (B). One microgram of total RNA was analysed by RT-PCR specific for primate (A) or mouse (B) erythropoietin receptor. The RT-PCR product for primate erythropoietin receptor is 485 bp, and that for the mouse EPO receptor is 452 bp. No RT, no reverse transcriptase added; positive control, human erythropoietin receptor cDNA; MEL, murine erythroleukaemia cells (positive control for murine erythropoietin receptor).

manuscript was under revision, showing hypoxia-inducible VEGF expression in rat astrocytes (Ijichi *et al.*, 1995). By contrast, complement C3 and contrapsin showed no significant effect upon stimulation by either stimulus. In conclusion, we showed that astrocytes expressed erythropoietin and VEGF in an oxygen-dependent manner and that cobalt chloride mimicked this up-regulation of VEGF but not of erythropoietin transcription.

#### *Putative physiological role of erythropoietin in the brain*

At present, the physiological function of erythropoietin in the nervous system remains to be clarified. By analogy to other ligands that bind to members of the cytokine receptor superfamily (including the erythropoietin receptor), erythropoietin might represent a polyfunctional growth factor. We recently proposed that the significant increase in erythropoietin mRNA in the brain upon hypoxic stimulation might represent a rapid adaptive mechanism of this organ to hypoxia (Digicaylioglu *et al.*, 1995). In this context, it is noteworthy that

erythropoietin and erythropoietin receptor mRNA expression was found in areas containing populations of neurons which are particularly susceptible to hypoxia, such as the hippocampus (CA1), neocortex and cerebellum (Haddad and Jiang, 1993). Furthermore, assuming that the erythropoietin receptor is present on neurons, as observed with neuron-like PC12 cells (Masuda *et al.*, 1993) and neuroblastoma cell lines (H. H. Marti, R. H. Wenger and M. Gassmann, unpublished observations), one could postulate that erythropoietin exerts a neurotrophic activity in the brain. In view of its role during erythropoiesis, erythropoietin might act as a survival factor preventing apoptosis as well as a differentiating factor during development. Interestingly, *in vivo* experiments have demonstrated that erythropoietin prolongs the survival of lesioned neurons after fimbria-fornix transections in rats (Konishi *et al.*, 1993). Furthermore, erythropoietin augmented choline acetyltransferase activity in mouse embryonic primary septal neurons (Konishi *et al.*, 1993) and increased monoamine and calcium levels in the pheochromocytoma-derived cell line PC12 (Masuda

*et al.*, 1993). During embryogenesis, erythropoietin might be involved in brain development: expression of erythropoietin and its receptor has been detected in the developing brain of 8- to 10-day old embryos (Yasuda *et al.*, 1993) as well as in blastocyst-derived embryonic stem cells and embryoid bodies, which resemble the 6–8 day egg cylinder stage (Schmitt *et al.*, 1991; Keller *et al.*, 1993; Gassmann *et al.*, 1995). Finally, oxygen-dependent expression of the cytokines erythropoietin and VEGF in astrocytes leads to the speculation that erythropoietin might play a role in angiogenesis in concert with VEGF; erythropoietin receptor is expressed on endothelial cells (Anagnostou *et al.*, 1994) and erythropoietin has been shown to exert mitogenic and chemotactic effects on endothelial cells (Anagnostou *et al.*, 1990) and to induce angiogenesis from rat aortic rings *in vitro* (Carlini *et al.*, 1995).

The view of erythropoietin as a monofunctional haematopoietic hormone, solely produced by the fetal liver and adult kidney and acting exclusively on erythroid precursor cells, needs to be revised: erythropoietin and its receptor are widely expressed throughout the body. A more defined role for erythropoietin in brain and other organs as well as during embryogenesis requires studies in transgenic mice containing the erythropoietin gene under the control of tissue-specific promoters.

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## Abbreviations

GFAP	glial fibrillary acidic protein
IL-6	interleukin-6
MEL	murine erythroleukaemia
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RT	reverse transcription
VEGF	vascular endothelial growth factor

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