Erythropoietin receptor signalling is required for normal brain development

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SUMMARY

Erythropoietin, known for its role in erythroid differentiation, has been shown to be neuroprotective during brain ischaemia in adult animal models. Although high levels of erythropoietin receptor are produced in embryonic brain, the role of erythropoietin during brain development is uncertain. We now provide evidence that erythropoietin acts to stimulate neural progenitor cells and to prevent apoptosis in the embryonic brain. Mice lacking the erythropoietin receptor exhibit severe anaemia and defective cardiac development, and die at embryonic day 13.5 (E13.5). By E12.5, in addition to apoptosis in foetal liver, endocardium and myocardium, the erythropoietin receptor null mouse shows extensive apoptosis in foetal brain. Lack of erythropoietin receptor affects brain development as early as E10.5, resulting in a reduction in the number of neural progenitor cells and increased apoptosis. Corresponding in vitro cultures of cortical cells from Epor−/− mice also exhibited decreases in neuron generation compared with normal controls and increased sensitivity to low oxygen tension with no surviving neurons in Epor−/− cortical cultures after 24 hour exposure to hypoxia. The viability of primary Epor+/+ rodent embryonic cortical neurons was further increased by erythropoietin stimulation. Exposure of these cultures to hypoxia induced erythropoietin expression and a tenfold increase in erythropoietin receptor expression, increased cell survival and decreased apoptosis. Cultures of neuronal progenitor cells also exhibited a proliferative response to erythropoietin stimulation. These data demonstrate that the neuroprotective activity of erythropoietin is observed as early as E10.5 in the developing brain, and that induction of erythropoietin and its receptor by hypoxia may contribute to selective cell survival in the brain.

Key words: Erythropoietin, Receptor, Brain, Heart, Development, Neural Progenitor Cells, Mouse

INTRODUCTION

Erythropoietin is a member of the haematopoietic cytokine superfamily that includes thrombopoietin, interleukin 3 (IL3), IL6, granulocyte-macrophage colony-stimulating factor (GM-CSF), prolactin, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT1), cytokines known to stimulate haematopoietic, neural or cardiac development. Erythropoietin receptor (EpoR) and other members of the corresponding cytokine receptor superfamily are characterized by polypeptides with a single transmembrane domain and an extracellular domain with a WSXWS motif (Youssoufian et al., 1993). During erythropoiesis, erythropoietin acts by binding to its receptor to stimulate the proliferation, differentiation and maturation of erythroid progenitor cells. Erythropoietin activates phosphorylation of Jak2/STAT5 proteins as well as other signal transduction pathways. Receptor homodimerization occurs upon erythropoietin binding, increasing the affinity of Jak2 for the membrane proximal region of the receptor, phosphorylating Jak2 and the cytoplasmic region of the receptor (Watowich et al., 1999).

Erythropoietin is required for the production of mature erythrocytes, the yolk sac being the first site of erythropoiesis (embryonic days 7-11 (E7-11)). In the embryo proper at E10, the aorta-gonad-mesonephros region gives rise to haematopoietic stem cells responsible for definitive haematopoiesis (Medvinsky and Dzierzak, 1996). By E12, the major site of erythropoiesis shifts to the liver. The effects of targeted deletion of erythropoietin receptor are dramatic when haematopoiesis shifts from the yolk sac to the foetal liver and definitive erythropoiesis resulting in combined failure of definitive erythroid progenitors to proliferate and differentiate into mature erythrocytes. Death
occurs at around E13.5 and is believed to result primarily from severe anemia that ensues in the foetuses (Lin et al., 1996; Wu et al., 1995). The inability of thrombopoietin, prolactin/prolactin receptor or IL6/soluble IL6 receptor to replace erythropoietin during erythropoiesis (Baiochi et al., 2000; Kieran et al., 1996; Socolovsky et al., 1998) shows that stimulation by the haematopoietic cytokines can be similar and may be more crucial for proliferative than for instructive signalling. This is particularly relevant to the production of EpoR in non-erythroid cells such as haematopoietic stem cells (Orlic et al., 1995), endothelium (Anagnostou et al., 1994; Ribatti et al., 1999), neuronal cells (Masuda et al., 1993; Morishita et al., 1997) and muscle (Ogilvie et al., 2000). EpoR is also required for normal embryonic heart development and vascularization. In addition to compromised erythropoiesis, Epor−/− embryos exhibit ventricular hypoplasia and disrupted vasculature in the heart prior to death (Wu et al., 1995). Myoblasts and primary satellite cells produce EpoR and exhibit a proliferative response to erythropoietin that can interrupt differentiation into myotubes in culture (Ogilvie et al., 2000). Administration of erythropoietin in vivo also stimulates neovascularization and/or blood vessel formation in endothelial tissues such as the chick embryo choorioallantoic membrane (Ribatti et al., 1999) and murine uterine endometrium (Yasuda et al., 1998). Furthermore, in adult brain, erythropoietin administration is neuroprotective against ischaemic damage in vivo (Bernaudin et al., 1999; Brines et al., 2000; Sakanaka et al., 1998). These observations provide evidence that erythropoietin activity is not restricted to the erythroid lineage.

We have previously indicated that the expression of erythropoietin receptor transcripts in mouse brain peaks at mid-gestation, then subsequently decreases to modest levels in the adult (Liu et al., 1994). Endogenous erythropoietin receptor expressed in cultured hippocampal and cerebral cortical neurons is functional and protects against glutamate-induced damage (Masuda et al., 1993; Morishita et al., 1997). Erythropoietin also stimulates the proliferation of neuronal NT2 cells exposed to hypoxia and increases the number of dopaminergic neurons in cultures of neuronal stem cells (Chin et al., 2000; Studer et al., 2000). In brain, production of erythropoietin has been detected in astrocytes and neurons (Juul et al., 1999; Masuda et al., 1994a), suggesting possible autocrine or paracrine regulation by erythropoietin. The induction of erythropoietin production by hypoxia suggests that erythropoietin may be particularly relevant in neuroprotection during hypoxia (Digicaylioglu et al., 1995; Juul et al., 1998; Masuda et al., 1994a). Animal studies of brain ischaemia show that erythropoietin administration provides protection against hippocampal CA1 neuronal damage and memory loss in gerbils (Sakanaka et al., 1998). In rats, erythropoietin has been shown to be neuroprotective in the cerebral cortex and to alleviate navigation disability caused by permanent occlusion of the cerebral artery (Sadamoto et al., 1998).

The current study evaluates the function of EpoR in the developing brain using mice with an Epor targeted deletion (Epor−/−) (Lin et al., 1996). We find that lack of EpoR signalling affects brain development as early as E10.5, resulting in a reduction of neural progenitor cells and increased apoptosis, and in a marked reduction in the survival of neuronal cell cultures exposed to hypoxia. The antiapoptotic effect of erythropoietin on embryonic neuron cultures is also observed. These studies provide evidence that EpoR signalling extends beyond erythropoiesis and haematopoietic activity and provides in vivo evidence that erythropoietin plays a role in neural progenitor cell survival and neuronal cell generation in the developing brain.

**MATERIALS AND METHODS**

**Production of Epor−/− mice**

Hemizygous mice for the targeted deletion of the Epor gene (Epor−/−) (Lin et al., 1996) were mated and embryos harvested from pregnant females at various stages of gestation. Embryos were genotyped by PCR analysis of DNA isolated from yolk sac. The PCR primers used to detect the null EpoR were: P1, GCC CCC TCT GTC TAC TT; P2, CGC CTC AAA ACC AGA AAC AG; P3, GAA GAG CCT GGC GCG GAA TG (Lin et al., 1996).

**Production of Epor-lacZ transgenic mice**

A hybrid reporter gene was constructed consisting of the Epor 5′ proximal promoter fragment extending from −1778 to −150 bp 5′ to the first codon linked to the lacZ coding region, and transgenic mice generated as previously described (Liu et al., 1997). Embryos were harvested and fixed in 1% formaldehyde, 0.2% glutaraldehyde, 2 mM MgCl2, 5 mM EGTA and 0.02% NP-40 in PBS for 1 hour at 4°C. After three washes in PBS plus 0.02% NP-40 for 30 minutes each, embryos were stained overnight at 37°C in solution with 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mg ml−1 X-gal. Stained embryos were fixed and embedded in paraffin for sectioning. Nuclear fast red was used as a counter stain.

**Immunohistochemistry**

Embryos were fixed in 10% formalin, embedded in paraffin and sectioned. Sections were deparaffinized in xylene. Immunostaining was carried out using polyclonal anti-EpoR (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:400 at 4°C overnight. Tenfold excess blocking peptide was used to determine the specificity of the primary antibody. Antibody binding was visualized using a biotinylated secondary antibody and an avidin/biotinylated peroxidase complex (ABC) (Vector Laboratories, Burlingame, CA). Sections were counter stained with haematoxylin for visualization. Polyclonal antibody for von Willebrand (Factor VIII) (Dako, Carpinteria, CA) was used at a 1:1600 dilution. For immunocytochemistry of murine cortical cells, antibodies and dilution were as follows: MAP2 1:500 (Sigma, St. Louis, MO), β-tubulin type III (TuJ1) monoclonal 1:500 (Babco, Richmond, CA), nestin (Rat 401) 1:1000 (BD PharMingen, San Diego, CA) and fluorescent labelled secondary antibody. Cell nuclei were counterstained with 0.001% DAPI (Sigma, St. Louis, MO).

**TUNEL assay**

For terminal-deoxynucleotidyl transferase (TdT)-mediated dUTP-end-labelling (TUNEL) analysis of apoptosis (Gavrieli et al., 1992), sections were treated with protease K and processed in a reaction mixture buffer containing digoxigenin-labelled dUTP and TdT (Roche Molecular Biochemicals, Indianapolis, IN), incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (AP) (Dako, Carpinteria, CA), visualized with a New Fuchsin substrate reaction and counter stained with haematoxylin. Sections incubated with the TUNEL reaction mixture but without TdT were served as negative controls. For neuronal cells, cultures were fixed in cold, freshly prepared 4% paraformaldehyde solution for 1 hour at 25°C and washed with PBS three times. After a 2 minute incubation in...
0.1% Triton X-100 and 0.1% sodium citrate, the cultures were washed three times with PBS and then incubated in the TUNEL reaction mixture for 2 hours at 37°C. Samples were analyzed under a fluorescence microscope. The proportion of apoptotic primary cortical neurons was quantified by counting the number of neurons with and without fragmented DNA in premarked microscope fields.

**Cell culture**

Human neuronal NT2 cells (Pleasure et al., 1992) were maintained in Opti-MEM I (Life Technologies, Gaithersburg, MD) with 10% or 2% foetal bovine serum with 5% CO2 and 20% or 2% oxygen tension. For isolation of cortical cells, the cortex from E10.5 embryos was dissected out and placed into neurobasal dissection media (1× Hank’s balanced salt solution (HBSS) with 10 mM HEPES, 0.3% glucose, 0.72% sucrose, pH 7.2). The tissue was digested with papain (7 units ml⁻¹ with 2.2 mM cysteine and 1 mM EDTA in dissection media) for 15 minutes at 37°C. The tissue was washed to inactivate the papain and triturated with a glass Pasteur pipette. The cells were counted using trypan blue and were plated at a density of 2×10⁵ cells per well in neurobasal medium with B27 supplement (Life Technologies, Gaithersburg, MD) onto poly-L-ornithine/fibronectin-coated glass coverslips in 24 well tissue culture plate. Four days after plating, cells were fixed and stained with MAP2 or β-tubulin type III, and TUNEL. Primary cultures of rat embryonic cortical neurons were prepared from E18 rat brains (Hampson et al., 1998; Priestley et al., 1990). The cortices were dissected and incubated for 10 minutes in a solution of 0.05% DNHase I and 0.25% trypsin (Life Technologies, Gaithersburg, MD). The sample was treated with 0.5% soybean trypsin inhibitor (Sigma, St Louis, MO) in HBSS (Life Technologies, Gaithersburg, MD) to stop digestion. Cells were dissociated by gentle trituration using Pasteur pipettes and resuspended in neurobasal medium (NBM) with the B-27 supplement (Life Technologies, Gaithersburg, MD), glutamine, and glutamate. Cells were initially plated at a density of 3.2×10⁴ per well in polylysine-coated 6-well plates. The neuronal content of the primary cultures was verified by intense immunohistochemical staining of the cultures with anti-neurofilament protein (a neuronal marker) and little staining with anti-glial fibrillary acidic protein (a marker for astroglial cells). Antibodies were obtained from Roche Molecular Biochemicals (Indianapolis, IN). On day 8, cells were subjected to supplement starvation upon exchange of the media for Locke’s solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, 5.0 mM glucose and 5.0 mM HEPES buffer, pH 7.2) (Chen et al., 1999) and cultured with 5% CO₂ and 20% O₂ or 2% O₂ for 24-48 hours. Cell survival was quantified by counting the total number of viable cells using trypan blue or by counting the number of undamaged neurons in premarked microscopic fields. TUNEL assay was used for the detection of apoptotic nuclei.

**RNA isolation and quantitative RT-PCR analysis**

Total RNA was isolated from neuronal cultures using STAT-60 (Tel-Test, Friendswood, TX) treated with RNase-free DNase (Promega, Madison, WI) at 5 units per 100 µg of RNA at 37°C for 30 minutes, followed by phenol-chloroform extraction and ethanol precipitation. First-strand cDNA was synthesized using MuLV reverse transcriptase and oligo-(dT)₁₆ (PE Applied Biosystems, Foster City, CA). Quantitative real-time RT-PCR analyses were carried out to determine the level of expression with the use of gene-specific primers and fluorescent labelled Taqman probes or SYBR green dye (Molecular Probes, Eugene, OR) in a 7500 Sequence Detector (PE Applied Biosystems, Foster City, CA) (Ogilvie et al., 2000). The probe was designed to span exon junctions in order to prevent the amplification of any contaminating genomic DNA. Serial dilutions of plasmid containing the cDNA of interests were used as template to determine a standard curve. All results were normalized with mouse S16 (mS16), rat S16 or human β-actin. The human EPO primer sequences used were as follows: forward primer, 5′-GCT CCC TTT GTC TCC TGC T-3′; reverse primer, 5′-CTC GAA ACA CAC CAA GTC CT-3′; probe, 5′-AGC GGC CTT GCT GCC GG-3′. The rat Epor primer sequences were as follows: forward primer, 5′-GAG AAT GAG TTT GAG GGP TTC TCC AT-3′; reverse primer, 5′-CCT CTA GGT GGG CAG GTG G-3′; probe, 5′-GGG TAA CTT CCA GCT ATG GCT GTT GCA AC-3′. The human GATA3 primer sequences were as follows: forward primer, 5′-CGG CTT CGG ATG CAA GTC-3′; reverse primer, 5′-GTC GAC GGT GCC CAA CAG-3′; probe, 5′-AGG CCC GGT GCA GCA CAA GAG G-3′. The human β-actin primers were as follows: forward primer, 5′-CTT GCC ACC CAG CAC AAT-3′; reverse primer, 5′-GCC GAT CCA CAC GGA GTA CT-3′; probe, 5′-TCA AGA TTA TTC CTC CTC ATG GC-3′. The mouse nestin primers were as follows: forward primer, 5′-GCC TAC ATA CAG GAT TCT GCT GG-3′; reverse primer, 5′-CAG GAA AGC CAA CAG AAG CCT-3′. Rat Bcl-x₇: forward primer, 5′-ATC GAG AAC GGC GCC GG-3′; reverse primer, 5′-GGC TCT CGG GTG CTG TG-3′; probe, 5′-TGC GAC ACT TTT GTT GAT CTC TAC GGC A-3′. Rat Epo: forward primer, 5′-TAG GTA CAT CTT GGA GCC CAA GAA-3′; reverse, 5′-TTC TCA CTC AGT CTG GGA CCT TC-3′; probe, 5′-GCA GAA AAT GCA ATG GCC GTG TG-3′. The sequences of mS16 and GATA3 primers and probes have been described previously (Ogilvie et al., 2000).

**Immunoprecipitation and western blotting**

For immunoprecipitation experiments, primary cortical cells cultured for 8 days were changed to Locke’s solution for 16 hours under 20% or 2% oxygen tension, then were exposed to 5 U ml⁻¹ of erythropoietin (Amgen, Thousand Oaks, CA) for 30 minutes. Cells were washed with cold PBS twice and then lysed in modified RIPA buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate and protease inhibitors). Whole cell lysates (1 mg) were incubated with 4 µg of anti-phosphoryrosine 4G10 antibody (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. Protein A agarose-captured immunocomplexes were separated on 4-12% Novex Bis-Tris NuPAGE Gels (Invitrogen, Carlsbad, CA) and transferred to PVDF membrane (Invitrogen, Carlsbad, CA) using a Novex Xcell II Mini-Cell and Blot Module (Invitrogen, Carlsbad, CA). Membranes were probed with anti-Jak2 at 1:1000 dilution (Upstate Biotechnology, Lake Placid, NY) and anti-STAT5 at 1:250 dilution (BD Transduction Laboratories, Lexington, KY) followed by horseradish-peroxidase (HRP)-coupled secondary antibodies and developed by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ).

**Transfection into NT2 cells**

The Epor proximal promoter with or without GATA binding sites linked to a luciferase reporter (Chin et al., 1995) was transfected into NT2 cells using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) in the presence or absence of a human GATA3 expression vector. Luciferase activity was determined 48 hours after transfection. For stable overexpression of GATA3, human GATA3 cDNA was cloned into the pIRE2-EGFP expression vector (Clonetech Laboratories, Palo Alto, CA). The GATA3 construct was transfected into NT2 cells using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Transfected cells were grown in selection medium containing G418 (Life Technologies, Gaithersburg, MD) for 2 weeks and harvested for analysis. Cells transfected with pIRE2-EGFP without insert were used for control.

**Statistics**

Data are expressed as means plus or minus standard error of the mean (SEM). Significance of differences was examined using the Student’s t test. P values <0.05 were considered to be significant.
RESULTS

Apoptosis in the Epor<sup>−/−</sup> foetal liver and heart

EpoR is required for the proliferation and maturation of definitive erythroid progenitor cells. Mice lacking endogenous EpoR (Epor<sup>−/−</sup>) die at E13.5 owing to interrupted definitive erythropoiesis (Lin et al., 1996; Wu et al., 1995). Normal primitive erythropoiesis is present in the Epor<sup>−/−</sup> embryo (Lin et al., 1996). In the Epor<sup>−/−</sup> mouse embryo, the foetal liver, site of foetal haematopoiesis, exhibited a marked increase in apoptotic cells including apoptotic hepatocytes and a reduction in the cell mass (Fig. 1A). In the normal mouse with productive erythropoiesis in the foetal liver, little apoptosis was seen (Fig. 1B).

In addition to being required for definitive erythropoiesis, Epor expression plays a role in normal heart development. No gross morphological differences in the hearts of Epor<sup>−/−</sup> and Epor<sup>+/+</sup> embryos were detected at E10.5. Starting from E11.5, ventricular hypoplasia was noted in the mice lacking endogenous EpoR and appeared to be related to ineffective cell proliferation and expansion of myocardium (Wu et al., 1999). Lack of staining for Epor transcripts in myocardium by in situ hybridization suggested that the erythropoietin effect on myocardium development was indirect. Our data suggest alternatively that Epor transcripts in myocardium may have been below the level of detection.

Using immunohistochemistry, we observed staining of EpoR in control heart at E11.5 that was localized to the pericardium,

![Fig. 1. Analysis of embryonic liver and heart. Foetal livers from Epor<sup>−/−</sup> (A) and Epor<sup>+/+</sup> (B) embryos at E12.5 were analysed by TUNEL. Apoptotic cells are stained red. (C-E) E12.5 embryos from Epor<sup>−/−</sup> mice were analysed for EpoR production in heart. Immunostaining of EpoR was detected in myocardium and endocardium (C,D); tenfold excess of blocking peptide completely blocked anti-EpoR antibody staining (E). (F,G,I,J) Von Willebrand factor (vWF) staining of E12.5 hearts Epor<sup>+/+</sup> mice (F,G) and Epor<sup>−/−</sup> (I,J) mice. Arrowheads indicate vWF positive cells. (H,K) TUNEL assay of Epor<sup>−/−</sup> (H) and Epor<sup>−/−</sup> (K) embryonic hearts show increased apoptosis in the Epor<sup>−/−</sup> embryo. Arrowhead indicates TUNEL positive cells. Bars, 0.025 mm (A,B,G,H,J,K) or 0.1 mm (C-F,I).]
myocardium, and endocardium (Fig. 1C,D). A marked reduction in von Willebrand factor positive endothelial cells was observed in the heart at E12.5 of Epor\(^{-/-}\) mice (Fig. 1I,J) compared with the normal control (Fig. 1F,G). We measured TUNEL staining in the hearts of control and Epor\(^{-/-}\) mice in order to determine whether the decreased cell proliferation caused by the lack of EpoR is related to loss of anti-apoptotic effect of EpoR signalling, resulting in an increase in apoptosis. A TUNEL procedure was used to identify the apoptosis in situ (Fig. 1H,K). In the heart of Epor\(^{-/-}\) mice at E11.5, there was significant increase of TUNEL-positive nuclei in the myocardium and endocardium (Fig. 1K). By comparison, only occasional TUNEL-labelled nuclei were observed in the heart of control animals (Fig. 1H). The defects in the embryonic heart appear in the formation of both the compact zone (epicardial region) and the trabeculae. Thus, cardiac myocytes in both regions may require erythropoietin signalling for survival/proliferation. This is consistent with EpoR localization in the normal embryonic heart (Fig. 1C,D). EpoR production in normal embryos and increased apoptosis in Epor\(^{-/-}\) embryonic heart are specifically localized. Lack of EpoR staining in the endocardial cushion (Fig. 1D) is consistent with lack of cushion defects in the null mutant.

**EpoR production in embryonic brain**

We and others have shown production of EpoR in brain (Digicaylioglu et al., 1995; Juul et al., 1999; Liu et al., 1997; Liu et al., 1994; Marti et al., 1996; Yasuda et al., 1993) with particularly high levels in the embryonic brain during midgestation (Liu et al., 1997; Liu et al., 1994). Immunostaining for EpoR was performed in sections of wild-type embryos at different stages. At E10.5, EpoR staining was broadly present in the all layers of neuroepithelia in forebrain, including the cortex, midbrain and hindbrain (Fig. 2A). By E11.5, EpoR was expressed most prominently in neuroepithelia of midbrain and hindbrain (Fig. 2B-F). In midbrain, EpoR production was localized to the intermediate zone cells (Fig. 2C). In hindbrain,
EpoR was produced in intermediate zone and ventricular zone (Fig. 2F). EpoR production in neuroepithelium of the forebrain was still present, although it is reduced. It was present in the subventricular and ventricular zones of cortex (Fig. 2D). EpoR immunoreactive cells were distributed through the intermediate zone of septum and ganglionic eminence (GE) (Fig. 2E). By E12.5, strong staining was mainly localized to the intermediate zone of pons and medulla of the hindbrain region (Fig. 2G).

During development, Epor–/– mice exhibited neuroepithelial tissue hypoplasia in the region of the pontine fixture adjacent to the fourth ventricle by E11.5, and this became further exaggerated at E12.5 (Fig. 2I) compared with the normal control (Fig. 2H). The degenerative changes in the Epor–/– nervous system were confirmed by TUNEL assay. As early as E10.5, Epor–/– mice showed increased apoptotic cells in the cerebral cortex (data not shown) and neuroepithelium of the midbrain (Fig. 2J,K) that was absent in the normal control (Fig. 2N,O). Marked increases in TUNEL-positive cells with increasing age was observed in regions of neuroepithelial tissues associated with EpoR production in Epor+/* embryos (Fig. 2L,M). The ganglionic eminence that exhibited EpoR staining by E11.5 in Epor+/* embryos also showed increased TUNEL-positive nuclei in Epor–/– mice (data not shown).

We have previously observed the highest level of endogenous EpoR production and human EPOR transgene expression in the brain at E10.5 (Liu et al., 1997). To study the function of EpoR signalling during neurogenesis, we examined primary cell cultures of Epor+/–, Epor+/- and

Fig. 3. Neuronal progenitor cells from Epor–/– cortex. (A) At E9.5, nestin expression is comparable in cortices of the Epor+/* and Epor+/- (n=5), and Epor–/– embryos (n=3). (B) Single-cell suspension isolated from E10.5 Epor–/– cerebral cortices (n=6) showed marked reduction in total cell numbers compared with Epor+/* and Epor+/- cortices (n=13). (C) By E10.5, nestin mRNA level was downregulated by four times in the Epor–/– cortex (n=4) compared with Epor+/* and Epor+/- (n=6). (D,E) A marked decrease in the numbers and proportion of nestin positive progenitor cells acutely isolated from the E10.5 Epor–/– cortex (n=4) was also observed compared with Epor+/* and Epor+/- cortices (n=12). (F,J) Fewer neurons were produced from in vitro cultures of Epor–/– cortical cells. Cells were stained for MAP2 (F) or β-tubulin III (red) and with DAPI (blue) after 4 days of culture in NBM. The proportions of cells with MAP2 positive staining are indicated in (F) for Epor–/– (n=5) and for Epor+/* and Epor+/- (n=6). Representative fields for Epor+/* cultures with 41% of 200 cells (J) and for Epor+/- cultures with 23% of 199 cells (K) with β-tubulin III positive staining are shown. (G-L,M) EpoR production improved neuronal cell survival under hypoxia. Six and 24 hours after cells were switched to Locke’s solution and cultured under the hypoxic condition of 2% oxygen tension, cells were stained with MAP2 (red), TUNEL (green) and DAPI (blue). After 6 hours, the number of surviving neurons was markedly decreased (G) and apoptotic neurons were significantly increased (H) in Epor–/– cultures (n=3). No surviving Epor–/– neurons were observed after 24 hours exposure (n=3) (G). About 10% of the surviving cells were neurons in the Epor+/* cultures. Erythropoietin addition increased the survival of Epor+/* neurons producing EpoR (I); no erythropoietin response was observed in Epor–/– cultures. In all experiments (E-M), cells were initially plated at the same density. For cell enumeration, the percentage of nestin (E) and MAP2 (F-I) positive cells isolated for each individual embryo were determined by an investigator blind to the genotypes by quantifying 20 microscopic fields documented by digital camera images. A total of 2000-4000 cells were counted. Scale bars: 0.025 mm in L,M; 0.05 mm in J,K. (B-F) *P < 0.01. Open bars represent Epor+/* or Epor+/- and Epor–/– cultures, as indicated, and solid bars represent Epor–/– cultures.
Epor\textsuperscript{+/+} E10.5 cortical cells. Single-cell suspensions were collected from E10.5 cerebral cortices. The total number of cortical cells harvested from Epor\textsuperscript{+/-} embryos was half that from Epor\textsuperscript{+/+} embryos, whereas the number from Epor\textsuperscript{+/-} embryos was comparable to that from Epor\textsuperscript{+/-} (Fig. 3B). At E10, the murine cerebral cortex is a single layer of germinal neuroepithelium composed predominantly of cells producing nestin, an intermediate filament protein normally found in neural precursor cells (Frederiksen and McKay, 1988; Lendahl et al., 1990). At E9.5, we found no differences in nestin production in the cortex of the Epor\textsuperscript{+/-}, Epor\textsuperscript{+/-} or Epor\textsuperscript{+/-} embryos (Fig. 3A). By contrast, by E10.5, nestin production in Epor\textsuperscript{+/-} cortical cells fell by four times compared with that of Epor\textsuperscript{+/-} cells (Fig. 3C). The levels in the Epor\textsuperscript{+/-} cortical cells were comparable to those of Epor\textsuperscript{+/-} cells. These data suggest that, between E9.5 and E10.5, lack of EpoR signalling results in a marked decrease in the proportion of neural progenitor cells. This was further supported by direct examination of nestin positive cells acutely harvested from E10.5 cortex (Fig. 3D,E). The Epor\textsuperscript{+/-} and Epor\textsuperscript{+/-} embryos exhibited 71±1% nestin positive cells compared with 57±2% for Epor\textsuperscript{+/-} embryos. Additional evidence for reduction in the capacity for neuronal cell production in the Epor\textsuperscript{+/-} cortex was obtained by cultures of cortical cells harvested at E10.5. Cells isolated from Epor\textsuperscript{+/-}, Epor\textsuperscript{+/-} and Epor\textsuperscript{+/-} embryos were plated at the same density. After 4 days of culture, the numbers of total cells and MAP2 or β-tubulin-III immunoreactive neurons were determined. The Epor\textsuperscript{+/-} cultures contained 27% MAP2 immunoreactive neurons compared with 39.6% for Epor\textsuperscript{+/-} and 33.8% for Epor\textsuperscript{+/-} cultures (Fig. 3F). Under normoxic culture conditions, the proportion of TUNEL positive cells was similar in the three groups. During the hypoxia, erythropoietin production can be induced up to 100-fold in neurons. In erythroid cells EpoR signalling increases not only progenitor cell proliferation but also survival and differentiation, especially under hypoxia. To study the survival effect of EpoR signalling on Epor\textsuperscript{+/-} cortical neurons, Epor\textsuperscript{+/-} cortical cells were grown for 4 days in culture and then switched into trophic-factor-free Locke’s solution and cultured at 2% O\textsubscript{2} for an additional 6 or 24 hours. After 6-hour culture, the proportion of apoptotic Epor\textsuperscript{+/-} neurons was significantly higher than that of Epor\textsuperscript{+/-} neurons (Fig. 3H). The percentage of surviving Epor\textsuperscript{+/-} neurons was decreased from 39.6±1.7% to 28.5±2.4%, whereas the percentage of surviving Epor\textsuperscript{+/-} neurons was decreased from 26.5±0.9% to 14.3±1.3% (Fig. 3G). After 24 hours, most of the Epor\textsuperscript{+/-} cortical cells became apoptotic and no MAP2 positive neurons were observed (Fig. 3G,M). However, in the corresponding Epor\textsuperscript{+/-} cell culture, fewer apoptotic cells were observed and 10.1±0.9% of surviving cells were neurons (Fig. 3GL). The Epor\textsuperscript{+/-} neurons exhibited greater sensitivity and markedly lower survival to hypoxia treatment than Epor\textsuperscript{+/-} neurons. The erythropoietin effect on Epor\textsuperscript{+/-} and Epor\textsuperscript{+/-} cortical neurons under hypoxia was also studied. Erythropoietin addition increased Epor\textsuperscript{+/-} neurons survival after 24 hours of culture under hypoxia (Fig. 3I). In the presence of erythropoietin, 14.2±1.2% of surviving cells were neurons, compared with 10.1±0.9% in the absence of erythropoietin. However, no erythropoietin neuroprotective effect was observed in the Epor\textsuperscript{+/-} cell culture, and no neurons survived. These data indicate that EpoR signalling is important in neuronal cell survival and the relative effects are even greater under hypoxic challenge.

**Erythropoietin stimulation of neuronal cells**

Reduction in neural progenitor cells in the Epor\textsuperscript{+/-} brain is consistent with the reduction in neural cell survival and increased apoptosis owing to loss of viability associated with erythropoietin signalling. To examine the direct effect of erythropoietin on neural progenitor cell proliferation, we examined cultured human NT2 neuronal cells that produce EpoR and are erythropoietin responsive. NT2 cultures were exposed to reduced oxygen tension (2% O\textsubscript{2}) for three days and cell growth was monitored. Compared with control cultures, there was a modest decrease in cell growth by hypoxia. By contrast, cells cultured with erythropoietin (5 U ml\textsuperscript{-1}) and hypoxia showed an increase in cell number 1.4 times that of cultures without erythropoietin (Fig. 4A). We also observed that low oxygen tension induced EpoR production more than twofold (Fig. 4B). In erythroid progenitor cells, erythropoietin induces expression of the transcription factor GATA1, a zinc finger containing protein that activates several erythroid specific genes including Epor (Chin et al., 1995). Other GATA-like proteins such as GATA3, which is required for normal brain development (Pandolfi et al., 1995), show some structural similarity to GATA1 and bind to similar DNA motifs, but exhibit different temporal and tissue specificity (Ko and Engel, 1993; Merika and Orkin, 1993). We observed that NT2 cells produce GATA3 and that erythropoietin induces GATA3 production (Fig. 4C). We also found that stable transfection of an expression vector for GATA3 into NT2 cells resulted in increased production of EpoR (Fig. 4D). Although we detected GATA2 production in NT2 cells, no increase in EpoR production was observed when an expression vector for GATA2 was used (data not shown). The Epor proximal promoter has a GATA consensus binding site that is required for active Epor promoter activity in erythroid cells. We used transfection assays of reporter gene constructs to show that deletion of this GATA binding site significantly decreased Epor promoter activity in NT2 cells (Fig. 4E). Increased GATA3 production induced Epor promoter activity twofold. We also found that absence of EpoR signalling downregulated GATA3 in E10.5 Epor\textsuperscript{+/-} cortex (Fig. 4F).

To study Epor promoter activity in vivo, we used a hybrid transgene consisting of a human Epor long (1778) or short (150) promoter with an intact GATA binding site fused to a lacZ reporter gene. As early as E10, three of 1778 lines showed transgene expression broadly distributed in the neuroepithelium of midbrain and hindbrain, where endogenous Epor expression was present (Fig. 2A). At E12, transgene expression was observed in the intermediate zone of the neuroepithelium of pons and medulla around the fourth ventricle in two of the 150 lines with the short Epor promoter (Fig. 2G). These regions corresponded to endogenous Epor expression. The transgene expression was also active in the haematopoietic tissues of these 150 lines at this stage. These data provide evidence that the Epor promoter can drive Epor tissue-specific expression.

**Neuroprotective effect of erythropoietin in embryonic neuronal cultures**

Examination of primary embryonic cortical cell cultures
demonstrated that EpoR production was required for neuronal survival under hypoxia. When it binds to EpoR, erythropoietin stimulates neuron survival, but cortical cells from the mouse embryo were not enough for large-scale studies. To study further the effect of erythropoietin on differentiated embryonic neuron survival, cortical neurons were isolated from rat embryos at E18 and cultured in neurobasal medium (NBM). After 8 days in culture, cells were subjected to trophic factor withdrawal, the media changed to Locke’s solution and cells cultured for an additional 24 hours. Addition of erythropoietin (5 U ml–1) with the change into Locke’s solution resulted in an increase in cell viability by ~1.5 times (Fig. 5A). When incubation was carried out at reduced oxygen tension (2% O2), the effect was even greater (1.75 times increase). The
The protective effect of erythropoietin on these primary embryonic neurons was also seen as a reduction in percentage of TUNEL positive cells (Fig. 5B). Addition of erythropoietin decreased the TUNEL positive cells incubated in Locke’s solution by a factor of 0.80 and 0.70 when cells were cultured at 20% and 2% oxygen, respectively. The greater differences (P<0.05) observed at low oxygen tension provided evidence that erythropoietin signalling can be protective even in local regions of hypoxia or ischaemia. The anti-apoptotic protein Bcl-xL is a Bcl2 family member associated with the protective activity of erythropoietin (Silva et al., 1996). Although the alternate short splicing form, Bcl-xS, inhibits the ability of Bcl2 to reduce apoptosis when cells were supplement starved in the absence of erythropoietin (Fig. 6B,C). The differences were particularly significant at low oxygen tension providing evidence that erythropoietin-induced Jak2 and STAT5 tyrosine phosphorylation in cortical neurons under normoxic or hypoxic condition.

Initial cultures of embryonic cortical neurons in the presence of NBM were not sensitive to erythropoietin. No change was observed on the viability of cells with trophic factors present with and without erythropoietin added during the initial phase of culture (Fig. 6A). However, erythropoietin supplementation of NBM for 4 days prior to the switch to supplement starvation was neuroprotective. Pretreatment for 4 days with erythropoietin while cells were grown in NBM was sufficient to reduce apoptosis when cells were supplement starved in the absence of erythropoietin (Fig. 6B,C). The differences were particularly significant at low oxygen tension resulting in a 30% increase in numbers of neurons in each field with erythropoietin treatment compared with the control (P<0.05) after 24 hours. The Bcl-xL protein level was also induced in the cortical neuronal cultures by erythropoietin pretreatment (data not shown).

**DISCUSSION**

During erythropoiesis, erythropoietin binding to its receptor stimulates proliferation, survival, and differentiation of erythroid progenitor cells. Absence of erythropoietin or its receptor interrupts definitive erythropoiesis in the foetal liver (Lin et al., 1996; Wu et al., 1995) as well as development of other specific organ systems (Wu et al., 1999), and tissue defects extend beyond erythroid cell maturation. We describe here corresponding defects with Epor−/− embryos, specific production of EpoR in the pericardium, endocardium and myocardium of developing heart but not the endocardial cushion, possibly reflecting their distinct developmental origins (Fig. 1). We show explicitly that there is extensive thinning and apoptosis of the ventricular compact zone and trabecular zone, indicating a reduction in proliferation of the myocardium as well as endocardium by E12.5 in the Epor−/− heart, corresponding to regions of EpoR staining in the Epor+/+ embryo. These data provide evidence that erythropoietin acts as a viability factor during cardiac development and is necessary to prevent apoptosis and expansion or proliferation of myocardial and endocardial progenitor cells. The ability of erythropoietin to stimulate endocardium is consistent with earlier observations of EpoR production in endothelial cells of vascular or aortic origin and of stimulation of neovascularization (Anagnostou et al., 1990; Anagnostou et al., 1994). In addition to the effect on myocardium, erythropoietin also stimulates myoblasts of skeletal muscle origin (Ogilvie et al., 2000). Erythropoietin promotes the proliferation of primary muscle satellite cells and can interrupt myoblast differentiation to myotubes. The suggested role for erythropoietin in these varied tissues provides support for the hypothesis that, rather than being restricted to the erythroid lineage, erythropoietin acts more generally to stimulate select progenitor cells to proliferate and expand, and to prevent apoptosis during differentiation. These observations add new light to the detection of high levels of erythropoietin expression during embryonic brain development.
Increasing evidence suggests that erythropoietin signalling may play a role in stimulating the development of multiple organs. The production of EpoR in neuronal cells and their response to erythropoietin (Fig. 4, Fig. 5, Fig. 6) (Chin et al., 2000; Juul et al., 1998; Masuda et al., 1993; Morishita et al., 1997; Studer et al., 2000), as well as the production of erythropoietin in astrocytes and neuronal cells (Juul et al., 1999; Masuda et al., 1994a), provide evidence for erythropoietin activity in the brain. During development, EpoR production in the brain peaks at mid-gestation. We found that, in the absence of EpoR production in Epor–/– mice, increased apoptotic cells in the brain were first seen at E10.5 (Fig. 2).

Neuroepithelium hypoplasia became apparent by E11.5. The regions affected corresponded to high endogenous EpoR activity in Epor+/+ embryos. To demonstrate that this is not due to general hypoxia caused by severe anaemia and heart defects, we examined Epor–/– embryos at E9.5 and E10.5, prior to significant anaemia or heart defects in the embryo proper. As early as E10.5, reduction of neural progenitor cells in Epor–/– embryonic cortex was observed. Fewer neurons were generated from these cortical cells harvested from Epor–/– embryos and cultured in vitro compared to Epor+/+ neural progenitor cells (Fig. 3). By contrast, no differences were detected in cortical cells at E9.5 (Fig. 3A). These data suggest that erythropoietin receptor signalling plays a role in the establishing neural progenitor cell population during neurogenesis, particularly after E9.5. Loss of EpoR does not affect progenitor cells in general, but instead only those from select lineages or tissue types. For example, although the Epor–/– embryo is severely anaemic by E12.5, the numbers of colony forming units (CFU)-granulocyte/macrophage and CFU-megakaryocyte in culture from E12.5 Epor–/– foetal livers were normal (Lin et al., 1996).

Erythropoietin production on both sides of the blood-brain barrier is inducible by hypoxia (Digicaylioglu et al., 1995; Masuda et al., 1994b). During anaemic stress, in addition to increased erythropoietin production, we observed induction of EpoR in both haematopoietic tissue and brain (Liu et al., 1997). Increase of erythropoietin and induction of EpoR in haematopoietic tissue is consistent with the increased erythropoietic response to hypoxia or anaemic stress, and raise the possibility that, in the brain, erythropoietin may provide, in part, an analogous neuroprotective effect. Animal studies demonstrated that erythropoietin can be protective in the adult brain to ischaemic challenge, particularly when increased by direct administration (Brines et al., 2000; Sakanaka et al., 1998). We now show that low oxygen has a specific effect on neuronal cells to induce both erythropoietin and EpoR expression, and in the absence of EpoR expression, neuronal cell survival is markedly affected by hypoxic treatment. This suggests a corresponding neuroprotective role for erythropoietin receptor signalling during brain development.

By E12.5, as with embryonic heart and erythroid development, the Epor+/+ genotype is associated with significant apoptotic activity in the brain corresponding to regions of EpoR expression in Epor+/+ embryos (Fig. 2). The neuroprotective effect of EpoR signalling was already evident in cultures of E10.5 cortical cells. When E10.5 cortical cells were cultured under normal differentiation medium, there was no significant difference between the apoptosis observed in Epor+/+ and Epor–/– surviving neurons. Cortical neurons were cultured under hypoxia and in the absence of trophic factors to study the survival of neurons differentiated from cortical cells. We observed a significant increase in apoptosis of ~40% in Epor–/– neurons compared with <10% in Epor+/+ neurons after 6 hours. The proportion of surviving Epor+/+ neurons decreased to 0.7 after 6 hours and to 0.25 after 24 hours incubation. Corresponding cultures isolated from Epor–/– embryos were markedly more affected, with the proportion of surviving neurons decreasing to 0.5 after 6 hours and no neurons surviving after 24 hours of incubation (Fig. 3). Erythropoietin addition further increased the survival of Epor+/+ neurons but not the Epor–/– neurons. The neuroprotective effect of EpoR signalling was clearly evident in these E10.5 cortical cell cultures. EpoR production provides for neuron survival under hypoxia challenge. It also suggests that the neuroprotective effect of erythropoietin is mediated by its unique binding with EpoR. A specific survival role for erythropoietin and induction of EpoR by hypoxia were also demonstrated in cultures of cortical neurons from normal animals. These cultures showed increased viability and reduced apoptosis when cultured with supplemental erythropoietin and in the absence of trophic factors (Fig. 5, Fig. 6). In addition to erythropoietin expression in brain by astrocytes and neurons, the induction of EpoR production in the neuronal cultures when exposed to low oxygen tension suggests that the erythropoietin response during hypoxia challenge to the brain may involve both an upregulation of erythropoietin production and an increase in neuronal sensitivity to erythropoietin mediated by increasing EpoR production. These data support the hypothesis that erythropoietin can act as a survival factor for neurons and can play a role in stress response to hypoxia or ischaemia.

A principal function of erythropoietin is to rescue committed erythroid progenitor cells from apoptosis (Kelley et al., 1993; Koury and Bondurant, 1990). Erythropoietin binding to its receptor in differentiating haematopoietic cells activates Jak/STAT and other signal transduction pathways to control cellular proliferation, survival and specific gene expression. Erythropoietin also stimulates Jak2 phosphorylation in endothelial and muscle cells (Ogilvie et al., 2000; Ribatti et al., 1999), and we observed stimulation of Jak2 phosphorylation in cultured cortical neurons (Fig. 5F). Bcl-x prevents massive cell death in neurons and foetal liver and death in utero (Motoyama et al., 1995), and Bcl-xL has been implicated in antiapoptotic effects mediated by erythropoietin on erythroid progenitor cells (Silva et al., 1996). Erythropoietin was sufficient to induce Bcl-xL in primary neurons when cultured without trophic factors under low oxygen tension (Fig. 5), suggesting that the ability of erythropoietin to induce Bcl-xL expression may be among the critical factors in neuronal cell survival.

Analysis of transgenic embryos showed that EpoR promoter drives specific transgene expression in brain corresponding to regions of endogenous EpoR production during development (Fig. 2). GATA1 and SPI binding sites are primary features of minimal promoter activity in erythroid cells (Chin et al., 1995). During erythropoiesis, erythropoietin stimulation of erythroid progenitor cells induces the production of the transcription factor GATA1, which is required for erythropoiesis and transactivation of a many erythroid specific genes including those for globin.
EpoR itself (Chiba et al., 1991; Chin et al., 1995; Zon et al., 1991). The GATA family of zinc-finger transcription factors binds to similar consensus sequences (A/TGATAA/G) (Ko and Engel, 1993; Merika and Orkin, 1993). In the brain, EpoR expression is related in part to the role of GATA3. Targeting of GATA3 results in severe deformities of the brain (Pandolfi et al., 1995). We observed that erythropoietin could induce GATA3 production in NT2 cells (Fig. 4) and in other non-haematopoietic cells (Ogilvie et al., 2000). In addition, overproduction of GATA3 in NT2 cells increases EpoR production. These effects are analogous to the interaction between GATA1 and EpoR in erythroid progenitor cells. Reporter gene transfection assays showed that, in neuronal cells, the GATA consensus sequence in the Epor promoter was required for its expression and that GATA3 induced Epor promoter activity (Fig. 4). Mutating the GATA consensus sequence in the Epor promoter diminished transactivity of GATA3. Furthermore, an Epor promoter containing the GATA binding site was active in embryonic brain in regions corresponding to EpoR production (Fig. 2). Interestingly, during early neurogenesis at E10.5, GATA3 production was downregulated in the Epor–/– cortical cells. These data raise the possibility that the effects of erythropoietin reported here for developing brain and in other progenitor cells may be generally mediated in part via induced expression of the GATA-like transcription factors.

We present here evidence that erythropoietin plays a role in brain and neuronal development and that production of EpoR in neuronal tissues is required for erythropoietin to stimulate the survival and provide for proliferation of corresponding progenitor cells. The induction of erythropoietin and its receptor by hypoxia suggests that for erythropoietin administration has therapeutic potential for tissue damage from ischaemia or hypoxic challenge in the central nervous system (Bernaudin et al., 1999; Brines et al., 2000; Sakanaka et al., 1998) and demonstrate that the protective role of erythropoietin is more general and extends beyond haematopoiesis. Furthermore, the induction of EpoR by hypoxia in select progenitor cells allows for specificity of cell response to increases in erythropoietin levels. For example, in cultures of embryonic neural stem cells, hypoxia as well as erythropoietin stimulates the selective increase in dopaminergic neurons (Studer et al., 2000). Neuronal cells appear to share common regulatory elements with haematopoietic cells in the production of EpoR. The multiple-organ response to erythropoietin that includes haematopoietic, neuronal, endothelial and muscle cells may be a consequence of the similarities of respective differentiating stem cells (Bjornson et al., 1999; Gussoni et al., 1999; Jackson et al., 1999). The common effect of erythropoietin to promote cell viability and to provide for cell proliferation may relate to the plasticity of tissue derived stem cells, particularly to that of the neural stem cells, which can also contribute to haematopoietic and muscle tissue when transplanted in vivo (Bjornson et al., 1999; Galli et al., 2000). Therefore, insights obtained from haematopoietic progenitor cell differentiation may provide a window on neuronal cell differentiation.

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