Cytotechnology **31:** 177–182, 1999.

In vitro neuroprotective action of recombinant rat erythropoietin produced by astrocyte cell lines and comparative studies with erythropoietin produced by Chinese hamster ovary cells

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Received 1 May 1998; accepted 29 October 1998

Key words: astrocyte, Chinese hamster ovary cells, neuroprotective action, recombinant rat erythropoietin

Abstract

In the central nervous system, astrocytes produce erythropoietin (Epo) and neurons express its receptor. To examine whether or not the brain Epo protects the *in vitro* cultured neurons from glutamate-induced cell death, we established rat astrocyte cell lines containing the plasmid for production of recombinant rat Epo. Epo partially purified from the culture medium showed a neuroprotective effect similar to that of rat Epo produced by Chinese hamster ovary (CHO) cells. Comparison was made in some other properties between Epo produced by these astrocyte cell lines and that by CHO cells.

Abbreviations: Epo, erythropoietin; EpoR, erythropoietin receptor; CHO, Chinese hamster ovary; EF-1α, eucaryotic polypeptide chain elongation factor 1α; EIA, enzyme-linked immunoassay; CNS, central nervous system; MTT, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; sEpoR, soluble erythropoietin receptor; NMDA, *N*-methyl-D-aspartate

Introduction

Epo is a key factor in red blood cell production by activating proliferation and differentiation of late erythroid precursor cells (Wu et al., 1995; Lin et al., 1996; Krantz, 1991; Jelkmann, 1992; Youssoufian et al., 1993). Epo involved in erythropoiesis is produced by the kidney in adults and the liver in fetuses (Krantz, 1991; Jelkmann, 1992; Youssoufian et al., 1993). Epo is a heavily glycosylated protein. Human Epo has three *N*-glycosylation sites and one *O*-glycosylation site (Jacobs et al., 1985; Lin et al., 1985; Goto et al., 1988). Rat Epo also has three *N*-glycosylation sites, although *O*-glycosylation site is missing (Nagao et al., 1992). *N*-Glycosylation of Epo including sialylation is essential for the *in vivo* activity but not for the *in vitro* activity (Tsuda et al., 1990; Yamaguchi et al., 1991). Sialylation influences the affinity of Epo to EpoR; desialyated Epo has higher affinity to EpoR.

In addition to the erythropoietic function of Epo, we and others have reported that the brain has a paracrine Epo/EpoR system (Masuda et al., 1993, 1994, 1997; Digicaylioglu et al., 1995; Marti et al., 1996; Morishita et al., 1996, 1997; Liu et al., 1997); neurons express EpoR (Masuda et al., 1993; Morishita et al., 1996, 1997) and astrocytes produce Epo (Masuda et al., 1994, 1997; Marti et al., 1996). As the blood brain barrier prevents penetration of blood Epo into the CNS, the brain Epo/EpoR system is totally independent of the endocrine Epo/EpoR system in the adult erythropoiesis. We also found that the brain Epo contributes to neuron survival by protecting neurons from ischemic damage (Morishita et al., 1997; Sakanaka et al., 1998). Previously, we partially purified brain Epo from rat cultured cerebral cells and confirmed its stimulatory effect on proliferation of erythroid cells (Masuda et al., 1994), but the amount was too small for examination of its properties including its action on cultured neurons. In this study,

we established astrocyte cell lines harboring rat Epo cDNA and partially purified Epo from the culture medium. Some properties of astrocyte Epo including the *in vitro* neuroprotective activity were compared with those of rat Epo produced by CHO cell lines.

Materials and methods

Human Epo and EIA

Human recombinant Epo was produced and isolated as described previously (Goto et al., 1988, 1989). Epo was measured with a sandwich-type EIA using two monoclonal antibodies that bind murine and human Epos at different epitopes (Goto et al., 1989). sEpoR, an ectodomain of EpoR that is capable of binding to Epo, was produced as described previously (Nagao et al., 1992)

Construction of plasmids for expression of rat Epo

REpo1.4 containing full length rat Epo cDNA (Nagao et al., 1992) was cloned into the *Eco*RI site of pUC19. Rat Epo expression vector, pEFratEpo, was constructed by ligation of *Eco*RI-blunted *Bst*EII fragment of REpo1.4 into *Eco*RI-blunted *Not*I site of pEFneo (Ohashi et al., 1994). pEFratEpo also contains the neomycin resistance gene.

Cell culture

Astrocyte cell lines were maintained with Dulbecco's modified Eagle's medium containing 10% fetal calf serum. CHO cells were maintained with Minimum Essential Medium Alpha Medium containing ribonucleotides, deoxyribonucleotides and 10% fetal calf serum. Ep-FDC-P2 cells were maintained with RPMI1640 medium containing 10% fetal calf serum. Primary cultures of dissociated hippocampal neurons were prepared from the brains of 19-day Wistar fetal rats as described previously (Morishita et al., 1997). The cells were cultured under 5% CO_2, 21% O_2 and 74% N_2 atmosphere.

Astrocyte cell lines and CHO cell lines producing rat Epo

Rat astrocyte cell lines (D2 and D3) were prepared by introducing the expression plasmid for SV40 T-antigen gene into the primary cultured rat astrocytes (Masuda et al., 1994). SV40 T-antigen produced immortalized cells. For selection of the immortalized cells, neomycin resistance, which derived from the cotransfected plasmid, was used. (Masuda et al., 1994). Astrocyte cell lines were cotransfected with two plasmids by the use of Trans IT LT1 (Takara, Tokyo, Japan); one plasmid contained rat Epo cDNA and the other plasmid is p3'ss (Invitrogen Co., CA) to confer hygromycin resistance. CHO cells were transfected with pEFratEpo by the use of Lipofectamine (Life Technologies Inc., MA). The colonies resistant to 100 μg ml^{-1} hygromycin in astrocyte cell lines and to 400 μg ml^{-1} G418 in CHO cells were picked up and examined for Epo production by EIA. Cellular proteins were determined by a protein assay kit (Bio-Rad Lab., CA).

Purification of Epo from the cultured medium

Epo-producing cells were cultured in 175 cm^2 T-flasks and Epo in the spent medium (1 liter for each cell line) was partially purified with a gel (500 μl) on which Epo-directed monoclonal antibody, R2, (Goto et al., 1989) was fixed as described previously (Masuda et al., 1994; Kambe et al., 1998).

Glycosidase treatment

Rat Epo was desialylated or deglycosylated by the use of neuraminidase and glycopeptidase F, respectively. Desialylated Epo was prepared by digesting 250 ng Epo with 250 mU neuraminidase from *Arthrobactor urefaciens* (Nakarai tesque, Kyoto, Japan) at 37 °C for 48 h in 10 mM phosphate buffered saline pH 7.4. The *N*-linked carbohydrate chain was removed by incubating 200 ng Epo with 6 mU glycopeptidase F from *Flavobacterium meningosepticum* (Takara) at 37 °C for 48 h in 10 mM phosphate buffered saline pH 7.4.

Western blotting

About 100 ng Epo was loaded onto the 12.5% polyacrylamide gel. Epo protein was detected by Western blotting using rabbit anti Epo antiserum as described previously (Masuda et al., 1994).

Assay for biological activities of Epo

Stimulation of erythroid cell proliferation by Epo was assayed by the use of Epo-dependent growth of Ep-FDC-P2 cells as described previously (Masuda et al., 1994). The stimulation increased absorbance at

600 nm due to MTT (Sigma, St. Louis, MO, U.S.A.) reduction.

The neuroprotective effect of Epo was assessed as described previously (Morishita et al., 1997). Briefly, primary cultured hippocampal neurons were incubated in the Hibernate A/N2 medium (Life Technologies Inc.) containing 0.15 mM glutamate for 15 min in a CO_2 incubator. The medium was replaced with fresh Hibernate A/N2 medium without glutamate and the cells were cultured for a further 24 h. Epo was added into neuron cultures for 24 h before the onset of glutamate challenge. Epo was removed just before exposure of neurons to glutamate and Epo was not present in the further 24-h culture. The cells were stained with trypan blue; nonviable cells incorporate the dye. Total cells and stained cells were counted directly under a microscope or by examining microphotographs.

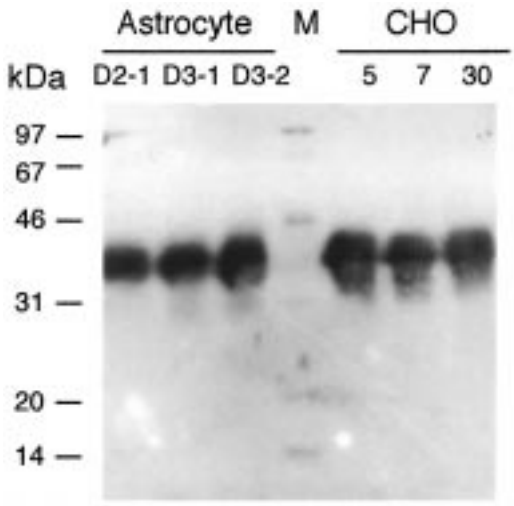

Figure 1. Western blotting analysis of Epo. Epo partially purified from culture media of astrocyte cell lines (D2-1, D3-1 and D3-2) and CHO cell lines (CHO5, CHO7 and CHO30) was detected by Western blotting analysis. M, molecular weight markers.

Results and discussion

Epo-producing cell lines and partial purification of Epo

As described in the Section 'Materials and methods', we prepared four rat astrocyte cell lines (D2-1, D2-2, D3-1 and D3-2) and three CHO cell lines (CHO5, CHO7 and CHO30), which harbored the plasmid containing rat Epo cDNA preceded by the EF1-α promoter and produced Epo in the culture medium. The yield Epo was 1.6 (ng/10^6cells/day), 1, 6, 9, 27, 27, and 24 for D2-1, D-2-2, D3-1, D3-2, CHO5, CHO7, and CHO30, respectively.

Epo in the culture medium was purified by affinity gel chromatography using Epo-directed monoclonal antibody. The eluted fraction was concentrated by ultrafiltration. Assay of Epo protein by EIA using recombinant human Epo as a standard indicated 100∼200-fold purification and 40–80% recovery of Epo protein. Based on the content of Epo protein in the total protein, the purity of the preparations was ∼10%.

Molecular size

Figure 1 shows rat Epo produced by astrocyte cell lines and CHO cells detected by Western blotting. Epo prepared from all CHO cell lines migrated with a molecular size of 35 kDa and Epo from astrocyte cell lines migrated with a slightly smaller size. As shown in Figure 2, neuraminidase treatment Epo yielded the product with a similar size of 30 kDa (Figure 2a) and the complete deglycosylation by digestion with glycopeptidase produced Epo with a molecular size of 18 kDa (Figure 2b), which is nearly equal to that calculated from the amino acid sequence based on cDNA (Nagao et al., 1992)

Biological activity

The stimulatory effect on the proliferation of an Epo-dependent erythroid cell line was used to assay the biological activity of Epo. As shown in Figure 3, Epo produced by astrocyte cell lines and CHO cell lines stimulated the cell proliferation in a dose-dependent manner, but there was no clear difference in the effectiveness of Epo produced by both cell lines. The Epo-dependent stimulation was completely blocked by the presence of sEpoR, which is a soluble form of EpoR capable of binding to Epo (Nagao et al., 1992).

L-Glutamate is a principal excitatory amino acid neurotransmitter in the mammalian CNS and also mediates pathological neuronal injury. Normally the increase of glutamate in the extracellular concentration in the CNS is limited to a very short period and in the spatially localized region appropriate for synaptic transmission, but a massive, sustained and unlocalized increase has been thought to be mainly responsible for neuronal death associated with reduction in oxygen (hypoxia) or glucose (hypoglycemia), or both (ischemia) (Benveniste et al., 1984; Choi, 1988; Choi and Rothman, 1990). At least three types of

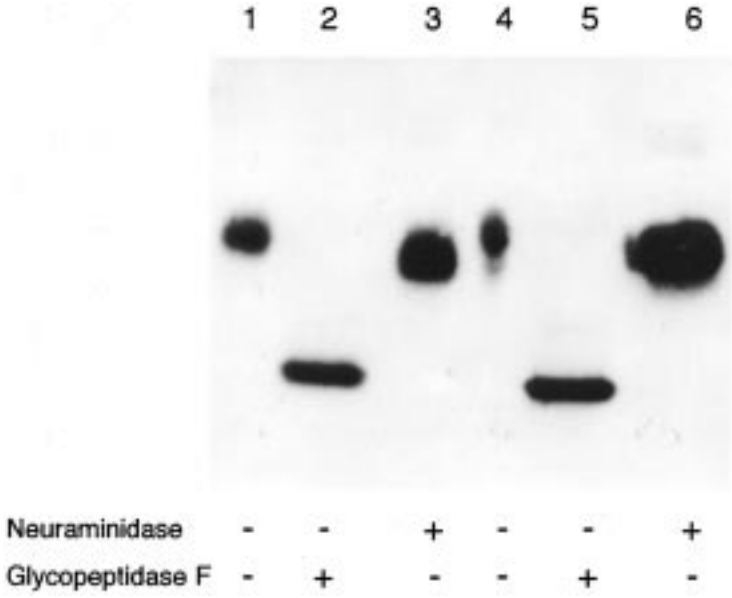

Figure 2. Digestion of Epo by neuraminidase and glycopeptidase. Epo was digested with neuraminidase or glycopeptidase F and detected by Western blotting analysis. Lanes 1–3, Epo from astrocyte cell line D3-1; lanes 4–6, Epo from CHO30. Lanes 1 and 4, untreated; lanes 2 and 5, digested with glycopeptidase; lanes 3 and 6, digested with neuraminidase.

membrane-spanning ionotropic receptors for glutamate have been found in neurons, each termed according to their agonists; NMDA, kinate, and quisqualate (Watkins and Olverman, 1987). Several pharmacological studies indicate that the NMDA receptor activation by binding with glutamate or agonists is directly involved in most glutamate neurotoxicity (Choi, 1988; Choi and Rothman, 1990). The NMDA receptor activation opens a channel permeable to both Na^+ and Ca^{2+}, while activation of two other receptors opens Na^+ channels. A lasting and massive increase of intracellular Ca^{2+} concentration evoked by glutamate-induced NMDA receptor activation plays a critical role in triggering intracellular events that elicit cell destruction (Choi, 1985, 1987; Siesjo, 1988) Dissociated and cultured neurons of the hippocampus and cerebral cortex have been used to investigate glutamate neurotoxicity *in vitro*, because both areas contain neurons vulnerable to ischemia-induced degeneration (Kirino, 1982; Pulsinelli et al., 1982). Previously, we found that recombinant human Epo protects the cultured hippocampal and cerebral cortical neurons from glutamate-induced death but Epo from a brain source was not tested. Thus, we examined whether or not the neuroprotective effect of astrocyte Epo differs from that of Epo derived from CHO cells. The cultured hippocampal neurons were incubated with glutamate for 15 min and the dead neurons were counted at 24 h after glutamate challenge. To examine the effect of Epo, Epo was added into the cultures 24 h before the glutamate challenge. Epos prepared from both types of cells prevented the glutamate-induced neuron death with similar effectiveness at a high concentration of Epo (30 pM) (Figure 4A), but Epo from astrocyte cell lines appears to be more effective at a low dose (3 pM) (Figure 4B).

Previously (Masuda et al., 1994), we found that brain Epo prepared from the cultured rat cerebral cells migrated with a molecular size of 33 kDa, which is smaller than that from rat serum, 35 kDa. The smaller size of brain Epo seems to be due to less sialylation than the serum Epo. Furthermore, the brain Epo had a higher affinity to EpoR of the erythroid cell line than the serum Epo did; brain Epo stimulated proliferation of erythroid cells at a low Epo concentration more efficiently than serum Epo did. Thus, Epo from astrocyte cell lines, which was thought to be equivalent to brain Epo from the cultured cerebral cells, might differ in the efficiency of the neuroprotective action from the CHO cell-derived Epo, which could be a substitute of serum Epo. Our experimental results, however, did not clearly indicate that Epo produced by astrocyte cell lines had a stronger neuroprotective effect than that produced by CHO cell lines, although astrocyte Epo in a low dose was somewhat more efficient. To idenfiy the cause of this unexpected finding, further compar-

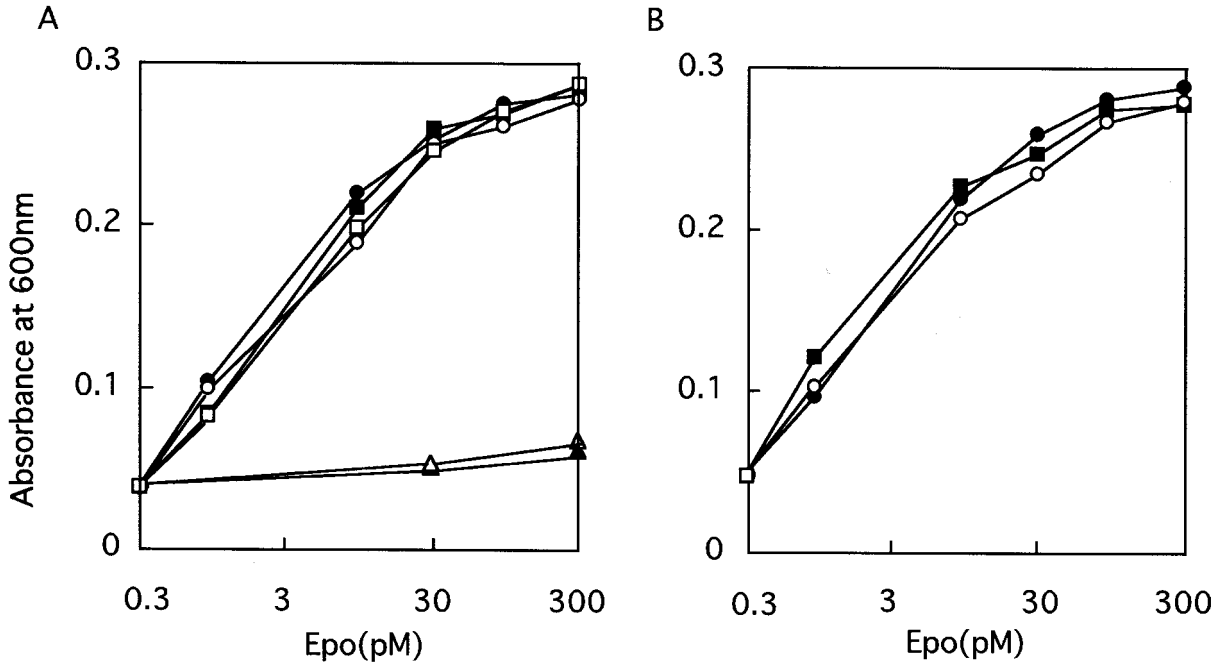

Figure 3. Stimulatory effect of Epo on proliferation of Epo-dependent erythroid cells. Stimulation of cell proliferation was measured as described in the Section 'Materials and methods'. In (A), Epo was partially purified from astrocyte cell line D2-1 (●), astrocyte cell line D2-2 (■), CHO5 (○), and CHO7 (□). Epo produced by D2-1 (▲) and CHO5 (△) was assayed in the presence of 100-fold sEpoR. In (B), Epo was prepared from astrocyte cell line D3-1 (●), astrocyte cell line D3-2 (■), and CHO30 (○). Each point indicates average value of triplicate assays.

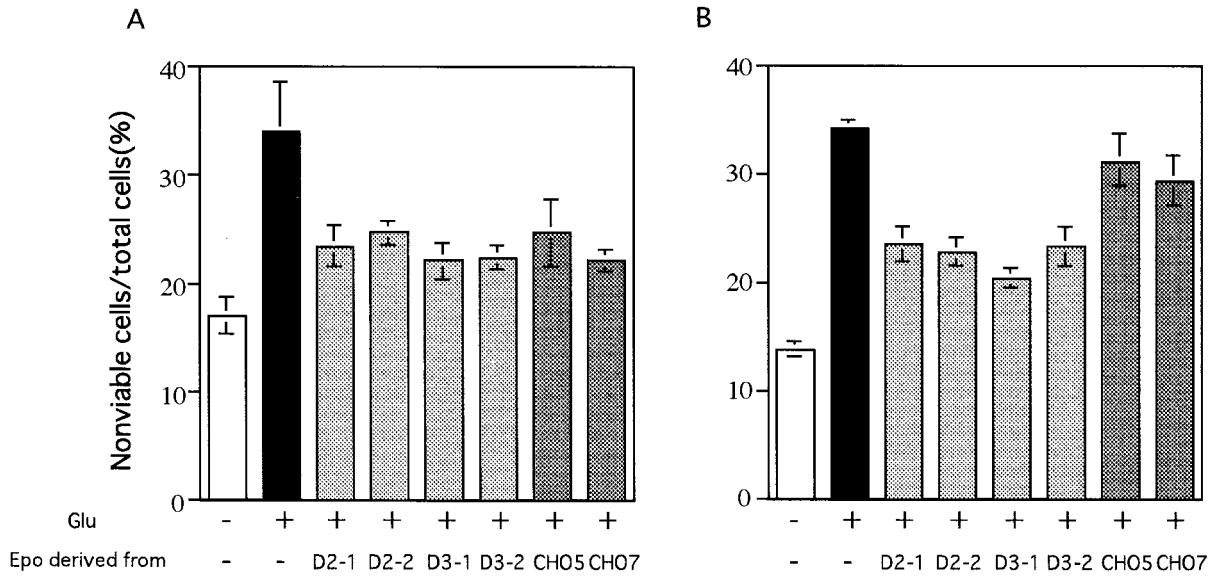

Figure 4. Protection of glutamate-induced cell death of cultured hippocampal neurons by Epo. Epo was added at 30 pM in (A) and 3 pM in (B). Glutamate (Glu) toxicity was examined as described in the Section 'Materials and methods'. Each column indicates the mean ± S.D. (n=3).

ison of Epo prepared from the cultured cerebral cells with Epo produced by astrocyte cell lines is necessary.

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