ELSEVIER

Journal of Neuroscience Methods 159 (2007) 8–18

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Inducible functional expression of Bcl-2 in human astrocytes derived from NTera-2 cells

Hakan Ozdener*

University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, United States

Received 20 April 2006; received in revised form 14 June 2006; accepted 14 June 2006

Abstract

Astrocytes provide structural support for neurons and may also play important roles in both neuroprotection and neurodegeneration. We, here report that human astrocytes derived from on NTera-2 (NT2) cell line expressing a functional anti-apoptotic protein bcl-2 under the control of a tetracycline responsive promoter using the Tet-On and Tet-Off expression systems. NT2 cells were transfected with the Tet On or Tet Off vectors followed by pTRE carrying human bcl-2. Drug resistant cells were differentiated into astrocytes under retinoic acid exposure. These astrocyte lines were found to express astrocyte specific markers such glial fibrillary acidic protein and chemokine receptors (CCR5, CXCR4) but not CCR3 and CD4. Furthermore, NT2 astrocytes expressing bcl-2 showed rapid response to doxycycline presence in the Tet On and Tet off system. The inducible expression of bcl-2 was found to be tightly regulated by doxycycline concentration in the NT2 astrocytes. We also showed that the induction of bcl-2 expression prevented NT2 astrocytes from camptothecin-induced cellular damage. These results indicate that this system may be useful for the study of specific effects of bcl-2 gene expression on astrocyte function(s) and cellular damage.

Keywords: Astrocytes; Bcl-2; NT2 cells; Apoptosis; HIV-1; Neurodegeneration

1. Introduction

Astrocytes, under normal conditions, play an important role in information processing by maintaining the extracellular concentration of glutamate at physiological concentrations. Beyond their role in brain development, astrocytes act as mediators of inflammatory responses and have significant roles in the mechanisms of neurodegenerative diseases such Alzheimer's disease and HIV associated dementia (Gorry et al., 2003; Xia and Hyman, 1999). Astrocytes are also supportive cells for neuron and other glial cells in the CNS. It has been shown that astrocyte and glial cell interaction enhance neuronal maturation, survival, synaptic transmission and axonal growth (Hartley et al., 1999). Primary neuronal and glial cells obtained from rodent and human fetal and adult tissues have been used for experimental purposes (Condorelli et al., 1997; De Groot et al., 1997; Ishii et al., 1992; Silva et al., 1998). Due to the limitation on obtaining human primary astrocytes, rat astrocytes are used as an alternative supportive layer and have provided valuable information about cell to cell interactions and the structure and function of the CNS (Aschner and Kimelberg, 1991; Muller and Seifert, 1982). However, the limited availability of these tissues encourages the establishment of immortalized cells and the utilization of stem cell cultures (Moe et al., 2005; Pilkington, 2005; Schuldiner et al., 2001). The NTera-2 (NT2) cell line is a homogeneous pluripotential cell line isolated from human teratocarcinoma that exhibits properties indicative of the committed neuronal precursor stage (Andrews et al., 1984). It has already been shown that NT2 cells can be differentiated into post mitotic central nervous system (CNS) neurons after retinoic acid treatment (Andrews, 1984). NT2 neurons are morphologically and functionally similar to mature human neurons, therefore they have been widely used as a model system to study neuronal development, function and mechanisms of cellular signaling and neuronal death (Chen et al., 2002; Guillemain et al., 2003). Recently, it was shown that NT2 cells could differentiate into astrocytes that express astrocytic markers such as glial fibriliary acidic protein (GFAP) and high affinity glutamate transporters after retinoic acid treatment (Bani-Yaghoub et al., 1999; Sandhu et al., 2002). Although undifferentiated NT2 cells that can be stably transfected then differentiated into neurons that over-express tranfected DNA but

* Present address: Monell Chemical Senses Center, 3500 Market Street, Philadelphia, PA 19104, United States. Tel.: +1 215 898 3260; fax: +1 215 898 2084.
E-mail address: hozdener@yahoo.com.

0165-0270/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2006.06.008

there has been no report of the use of astrocytes obtained from NT2 cell lines stably expressing a protein of interest. We, here, report a stable NT2 astrocytic cell line expressing bcl-2 protein under the control of a tetracycline responsive promoter using the Tet-On and Tet-Off expression systems. The increased expression of bcl-2 can protect NT2 astrocytes from apoptosis induced by camptothecin exposure.

2. Materials and methods

2.1. Construction of Bcl-2 F/pTRE expression vector

The human Bcl-2 in pSFFV (Splenic Follicular Focus Virus) was digested with *Eco*RI (Promega) and 1.9 kb full-length insert was gel cleaned (Stratagene) and ligated into *Eco*RI site of pTRE vector (Clontech) in 16 °C water bath for overnight. Ligated material was transformed into *E. coli* DH5a cells and then DNA obtained by mini prep kit (Promega) was screened with *Eco*RI. Positive clones were further screened by unique enzymes to determine forward and reverse orientation of Bcl-2 (bcl-2F/pTRE and bcl-2R/pTRE, respectively).

2.2. Transfection and establishment of NT2 Tet On/bcl-2F and NT2 Tet Off/bcl-2F cells lines

To establish NT2 cells expressing tetracycline response element, NT2 cells ($(2–3) \times 10^5$) were seeded into six wells plate the night before transfection. NT2 cells were transfected with either Tet-On (5 μg) or Tet-Off DNA (5 μg) using Lipofectamine 2000 (Stratagene) according to the manufacturer's instruction. Twenty-four to forty-eight hours after transfection, cells were maintained in DMEM medium supplemented with 400 μg/ml active G-418 for 1 month. On day 15, cells (NT2.Tet-On, NT2.Tet-Off) resistant to G-418 started to appear. In the second step, to establish inducible bcl-2 expressing stable cells, NT2 Tet On and NT2 Tet Off cells ($(2–3) \times 10^5$) were double transfected with bcl-2F/pTRE and pTK-Hyg DNA (Clontech) at ratio of 10:1 by using lipofectamine 2000. After 24 h, the medium was replaced with fresh medium supplemented with 200 μg/ml of hygromycine. Colonies that resistant to hygromycine were selected after an additional month of culture. Resistant colonies from each line were designated as NT2 Tet On/bcl-2F and NT2 Tet Off/bcl-2F. During this period, cells (NT2 On/bcl-2F and NT2 Off/bcl-2F) were maintained in either absence or presence of Doxycycline (1 μg/ml) to prevent induction of bcl-2 expression, respectively. Further, to determine doxycycline responsiveness for bcl-2 expression, both cell lines was analyzed by flow cytometry and Western blot. Cells showing response to dox were further used to obtain astrocytes expressing bcl-2 as described below.

2.3. Establishment and maintenance of astrocytes derived from NT2 cell lines

Undifferentiated NTera-2 cl.Dl (NT2, human embryonal teratocarcinoma) cells were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37 °C/5% CO_2. Astrocytes were derived from NT2 cells by modification of techniques described previously (Bani-Yaghoub et al., 1999; Sandhu et al., 2002). Briefly, 2×10^6 cells were seeded in a 75 cm^2 flask and exposed to 10 μM retinoic acid for 5 weeks. The cells were rinsed with sterile Versine twice and then trypsinized. The cells were dispersed into a single cell suspension for re-plating into nine tissue culture dishes (Falcon, 10 cm) in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml), 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine and 10 μM uridine (Sigma). Seven days later the neurons were separated by trypsin from the background cells, which were then maintained for an additional 10–20 days, during which time they demonstrated a flattened astrocytic morphology. These cells were then re-plated either into tissue culture wells or onto glass coverslips for immunofluorescence labeling and confirmation of GFAP expression.

2.4. Polimerase chain reaction (PCR)

The expression of transfected DNAs (pTet On and Off, pTRE and pTK-Hyg DNA) was confirmed in transfected NT2 cells by PCR using specific primers (Table 1). DNA was extracted from cells using Trizol reagent (Invitrogene) and PCRs were carried out with total volume of 50 μl, containing 10 mM Tris–Hcl (pH 8.0), 50 mM KCl, 1.5 mM $MgC1_2$, 0.01% (w/v) gelatin, 200 μM dNTPs, 20 pmol of each primer and 2.5 U of Taq Polymerase (Promega). Samples were subjected to 35 cycles of denaturing step at 94 °C for 1 min, an annealing step at 55 °C for 1 min and an extension step at 72 °C for 1 min 30 s. PCR product was visualized on 1.8% agarose gel.

2.5. Western blot

After 24 h of culture in the presence different concentrations of Dox (0.1–1 μg/ml), NT2 Tet-On/bcl-2F and NT2-Tet-Off/bcl-2F cells were rinsed with PBS and lysed into RIPA buffer containing protease inhibitors. Protein concentrations were determined by using a Bio-Rad Dc protein estimation kit. Equal concentrations of protein (25–40 μg/well) were run on either 12 or 6–16% gradient ready cast gels (BioRad) and proteins were blotted onto PVDF membranes. Membranes were blocked for 1 h at room temperature in 5% dried skim milk/PBS, and then incubated with either a rabbit anti-human Bcl-2 polyclonal antibody (SantaCruz; 1:500) or anti mouse cytochrome c antibodies (Pharmingen, 1:1000) for 1 h at room temperature. After five washes with PBS-T (Tween-20, 0.5%) for 10 min each, membranes were incubated with an HRP-labeled secondary antibody

Table 1
Primers for pTET-On/Tet-Off, pTRE and pTK-Hyg

pTet On/Off reverse	5′-ACGGGGATTTCCAAGTCTC-3′
pTet On/Off forward	5′-TGGTGATGCGGTTTTGGCAG-3′
pTRE reverse	5′-CGGGTTGGACTCAAGACGATAG-3′
pTRE forward	5′-AGAGGCGGTTTGCGTATTGG-3′
pTK-Hyg reverse	5′-GGGTGTGTTAGCAAACTACAGGACC-3′
pTK-Hyg forward	5′-TGAGAGGACATTCCAATCATAGGC-3′