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Transferrin receptor-1 suppresses neurite outgrowth in neuroblastoma Neuro2A cells

Yukary Nakamura [a,b], Noritaka Nakamichi [a], Takeshi Takarada [a], Kiyokazu Ogita [b], Yukio Yoneda [a,*]

[a] Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan
[b] Department of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

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ABSTRACT

Transferrin receptor-1 (TfR1) is a cell membrane-associated glycoprotein responsible for incorporation of the iron bound to transferrin through an endocytotic process from the circulating blood. Iron is believed to play a dual role as an active center of the electron transfer system in mitochondria and as an endogenous cytotoxin through promoted generation of reactive oxygen species in different eukaryotic cells. In this study, we evaluated expression profiles of different genes related to iron mobilization across plasma membranes in neuronal cells. Marked mRNA expression was seen for various iron-related genes such as TfR1 in cultured mouse neocortical neurons, while TfR1 mRNA levels were more than doubled during culture from 3 to 6 days. In mouse embryonal carcinoma P19 cells endowed to differentiate into neuronal and astroglial lineages, a transient increase was seen in both mRNA and corresponding protein for TfR1 in association with neuronal marker expression during culture with all-trans retinoic acid (ATRA). In neuronal Neuro2A cells cultured with ATRA, moreover, neurite was elongated together with increased expression of both mRNA and protein for TfR1. Overexpression of TfR1 significantly decreased the length of neurite elongated, however, while significant promotion was invariably seen in the neurite elongation in Neuro2A cells transfected with TfR1 siRNA as well as in Neuro2A cells cultured with an iron chelator. These results suggest that TfR1 would be highly expressed by neurons rather than astroglia to play a negative role in the neurite outgrowth after the incorporation of circulating transferrin in the brain.

1. Introduction

Transferrin receptor-1 (TfR1) is a transmembrane glycoprotein expressed on the cellular surface to mediate the incorporation of extracellular transferrin (Tf) in the circulating blood. The blood–brain barrier is composed of endothelial cells surrounded by basement membranes in which pericytes are found in a close apposition with astrocytic foot processes, while TfR1 is expressed on the lumenal membrane of endothelial cells. Transferrin is comprised of apo-transferrin (apo-Tf) and two molecules of ferric iron as an agonist for Tf receptors expressed at surfaces of different eukaryotic cells toward the endocytotic incorporation into endosomes, where ferric iron is reduced to ferrous iron by six-transmembrane epithelial antigen of the prostate-3 (STEAP3) (Bradbury, 1997; Moos and Morgan, 2000; Scheiber and Goldenberg, 1993; Ohgami et al., 2006). Ferrous iron is then liberated from apo-Tf in endosomes for the release into cytosol by divalent metal transporter-1 (DMT1), while apo-Tf is released into circulating blood through an exocytotic mechanism (Gunshin et al., 1997; Qian et al., 1997). Intracellular cytosolic ferrous iron is exported from the cell by ferroportin into extracellular spaces where ferrous iron is again oxidized to ferric iron by caeruloplasmin for the recognition by circulating apo-Tf (Bradbury, 1997; Wu et al., 2004).

In recent studies, iron has been shown to be drastically accumulated in particular brain regions in patients suffering from a variety of neurodegenerative diseases such as Alzheimer's disease (Ong and Farooqui, 2005; Quintana et al., 2006) and Parkinson's disease (Berg, 2006; Berg et al., 2006; Fasano et al., 2006a,b). Abnormal iron accumulation is also seen in the brains of patients with the

Abbreviations: α-MEM, alpha modified minimum essential medium; apo-Tf, apo-transferrin; ATRA, all-trans retinoic acid; Dcytb, duodenal cytochrome-b; DFO, deferoxamine; DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; DMT1, divalent metal transporter-1; FBS, fetal bovine serum; ferritin-H, ferritin-heavy chain; ferritin-L, ferritin-light chain; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; IRP1, iron regulatory protein-1; MAP2, microtubules-associated protein-2; PBS, phosphate-buffered saline; ROS, reactive oxygen species; STEAP3, six-transmembrane epithelial antigen of the prostate-3; Tf, transferrin; TfR1, transferrin receptor-1; ZIP14, zinc-regulated transporter/iron-regulated transporter-like protein-14; ZRT, zinc-regulated transporter.

* Corresponding author.
E-mail address: yyoneda@p.kanbazawa-u.ac.jp (Y. Yoneda).

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inherited neurodegenerative diseases with impaired iron homeostasis such as aceruloplasmia and neuroferritinopathy (Curtis et al., 2001; Xu et al., 2004; Gregory and Hayflick, 2005). The hypothesis that abnormal iron accumulation would lead to increased generation of reactive oxygen species (ROS) due to the reaction of ferrous iron with endogenously-evolved hydrogen peroxide to yield highly cytotoxic hydroxyl radicals is prevailing.

On the other hand, deferoxamine (DFO) is a high-affinity iron chelator approved as a first-choice medication for the clinical use in thalassemia (Cohen et al., 2004; Cianciulli, 2009). In addition, the European Medicines Agency recently approved DFO as an orphan drug for the treatment of traumatic spinal cord injury (European Medicines Agency, 2009). DFO forms a complex with iron to prevent the Fenton reaction, where ferrous iron catalyzes the production of hydroxyl radical along with hydrogen peroxide as described above (Gutteridge et al., 1979). In previous in vitro studies, DFO is also shown to scavenge hydroxyl radical (Hoe et al., 1982), to increase the differentiation to neurons from neural progenitor cells (Kim et al., 2006), to promote neurite outgrowth (Misumi et al., 2008) and to induce neurite outgrowth for synapse formation in postnatal rat dorsal root ganglion cell cultures (Nowicki et al., 2009). Moreover, the administration of DFO decreases the total hemorrhage volume in the ipsilateral hemisphere in rats with transient focal cerebral ischemia and hyperglycemia in vivo (Xing et al., 2009).

In the present study, therefore, we have attempted to elucidate the physiological and pathological significance of machineries required for cellular iron mobilization in the brain using mouse cortical neurons, mouse embryonal carcinoma P19 cells and mouse neuroblastoma Neuro2A cells in culture.

2. Materials and methods

2.1. Materials

An antibody against TfR1 was purchased from Invitrogen (Grand Island, NY, USA). ISOGEN was obtained from Nippon Gene Co. (Tokyo, Japan). rTaq DNA polymerase was purchased from Takara Bio, Inc. (Otsu, Japan). Dulbecco's modified Eagle's medium (DMEM), DMEM: Nutrient Mixture F-12 (DMEM/F-12) and alpha modified minimum essential medium (α-MEM) were provided by GIBCO BRL (Gaithersburg, MD, USA). Poly-L-lysine and cytosine arabinoside were from Sigma Chemicals (St. Louis, MO, USA). Mouse embryonal carcinoma P19 cells were supplied by ATCC (Manassas, VA, USA). Lipofectamine2000, lipofectamineRNAiMAX, Opti-MEM, small interfering RNA targeting the mouse TfR1 gene (siTfR1) and negative control (non-targeting siRNA) were supplied by Invitrogen (San Diego, CA, USA). The dual luciferase reporter assay system, pGL3-basic vector and pRL-SV40 vector were purchased from Promega (Madison, WI, USA). pEGFPC2 was from Clontech (Mountain View, CA, USA). All other chemicals used were of the highest purity available.

2.2. Culture of primary mouse cortical neurons

This study was carried out in compliance with the Guideline for Animal Experimentation at Kanazawa University with an effort to minimize the number of animals used and their suffering. Primary cortical neuronal cultures were carried out according to the method of di Porzio et al. (1980), with minor modifications (Hirai et al., 2005). In brief, cerebral cortices from 15-day-old embryonic ddY mice were dissected and incubated with Versene at room temperature for 12 min. Cells were mechanically dissociated by using a Pasteur pipette with a fire-narrowed tip in culture medium and plated at a density of 2.5×10^5 cells/cm^2 on plastic dishes coated with 7.5 μg/ml poly-L-lysine and 10% fetal bovine serum (FBS). Cortical neurons were cultured in DMEM/F12 containing 10% FBS and supplemented with 28 mM glucose, 2 mM glutamine, 5 mM HEPES, 0.11% sodium bicarbonate, 100 U/ml penicillin and 100 μg/ml streptomycin for the initial 24 h at 37 °C in a humidified 5% CO_2 incubator. Medium was then changed to DMEM/F12 containing 5% FBS with the aforementioned supplementation and with 25 μg/ml apo-transferrin, 250 ng/ml insulin, 0.5 pM β-estradiol, 1.5 nM triiodothyronine, 10 nM progesterone, 4 ng/ml sodium selenite and 50 μM putrescine. During 2–3 days in vitro (DIV), cells were treated with 5 μM cytosine arabinoside for 24 h to reduce proliferating cells. After 3 DIV, medium was changed to DMEM supplemented with 33 mM glucose, 2 mM glutamine, 5 mM HEPES, 0.11% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml apo-transferrin, 500 ng/ml insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/ml sodium selenite and 100 μM putrescine with medium change every 3 days. Under these culture conditions, approximately 98% of cells were immunoreactive for the neuronal marker microtubules-associated protein-2 (MAP2) on double immunocytochemical analysis using antibodies against MAP2 and glial fibrillary acidic protein (GFAP) (Hirai et al., 2005).

2.3. Culture of P19 cells

The pluripotent P19 stem cells derived from murine embryonal carcinoma differentiate into neuronal and astroglial lineages in the presence of all-trans retinoic acid (ATRA) as described previously (Rudnicki and McBurney, 1987). Prior to the treatment with ATRA, undifferentiated P19 cells were cultured at least three times in αMEM supplemented with 10% FBS. P19 cells were then plated onto 0.2% agarose coated dishes (φ100 mm) at a density of 1×10^5 cells/ml in αMEM supplemented with 5% FBS and 0.5 μM ATRA for proliferation, followed by culture for 4 DIV under floating conditions. These floating cells were harvested for trypsinization, followed by plating onto dishes previously coated with poly-L-lysine at a density of 2×10^5 cells/ml in αMEM supplemented with 10% FBS and subsequent culture for an additional 12 DIV in the absence of ATRA for induction of spontaneous differentiation. Culture medium was changed every 2 days, and cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

2.4. Culture of Neuro2A cells

Neuro2A cells derived from mouse neuroblastoma exhibited properties of neuronal stem cells to differentiate into neuron-like cells in the presence of ATRA. Undifferentiated Neuro2A cells were cultured in DMEM supplemented with 10% FBS and passed at least three times prior to the treatment with ATRA. Neuro2A cells were then plated on at 5×10^4 cells/ml in DMEM supplemented with 10% FBS for 24 h, followed by medium change to DMEM supplemented with 2% FBS and 20 μM ATRA for differentiation. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37 °C with medium change every 2 days.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA were extracted from liver, whole brain and cultured cells with the standard ISOGEN procedure and then subjected to the synthesis of cDNA. The individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, PCR buffer, dNTPs, relevant sense and antisense primers (Table 1), and rTaq DNA polymerase. Reactions were initiated by incubating at 94 °C for 10 min, and PCR (denaturation at 94 °C for 1 min, annealing at 58–65 °C for