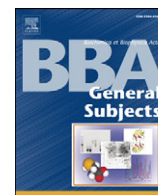
Biochimica et Biophysica Acta 1840 (2014) 2829–2842

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen



Roles of carbonic anhydrase 8 in neuronal cells and zebrafish

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ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 7 April 2014
Accepted 23 April 2014
Available online 1 May 2014

Keywords:
Carbonic anhydrase 8
SK-N-SH
Neuro-2a
Zebrafish
Cerebellar granule neuron
Cell migration and invasion

ABSTRACT

Background: Carbonic anhydrase 8 (CA8) is an isozyme of α-carbonic anhydrases (CAs). Previous studies showed that CA8 can be detected in human adult brain, with more intense expression in the cerebellum. Single mutations in CA8 were reported to cause novel syndromes like ataxia, mild mental retardation or the predisposition to quadrupedal gait.
Methods: In the present study, we examine the functions of CA8 in neuronal cell lines, mouse cerebellar granule neurons and zebrafish.
Results and conclusions: We demonstrated that overexpression of CA8 in neuronal cells significantly decreased cell death under staurosporine treatment. Moreover, CA8 overexpression significantly increased cell migration and invasion ability in neuronal cells and in mouse cerebellar granule neurons, implicating that CA8 may be involved in neuron motility and oncogenesis. By using zebrafish as an animal model, motor reflection of 3 dpf zebrafish embryos was significantly affected after the down-regulation of CA8 through *ca8* morpholino.
Conclusions: We concluded that CA8 overexpression desensitizes neuronal cells to STS induced apoptotic stress and increases cell migration and invasion ability in neuronal cells. In addition, down-regulated CA8 decreases neuron mobility in neuronal cells and leads to abnormal calcium release in cerebellar granule neurons. Knockdown of the *ca8* gene results in an abnormal movement pattern in zebrafish.
General significance: Our findings provide evidence to support that the impaired protective function of CA8 contributes to human neuropathology, and to suggest that zebrafish can be used as an animal model to study the biological functions of human CA8 in vivo.

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1. Introduction

Carbonic anhydrase 8 (CA8) is one of the 3 catalytically inactive carbonic anhydrase isozymes (CA8, 10 and 11), also known as carbonic anhydrase-related proteins (CA-RPs), which lack the function to catalyze the reversible hydration of CO_2 due to the lack of zinc binding histidine residues [1,2]. Previous study showed that the sequence of CA8 has 97.9% identity at the amino acid level between human and mouse [3]. Phylogenetic study also showed that the protein sequence identities of CA8 ranged from 67% to 100% among different vertebrates like *Bos taurus*, *Canis familiaris*, *Danio rerio*, etc. [4], suggesting that CA8 may play biologically important roles in higher organisms. In human adult brain, CA8 was distinctly expressed in the neural cell body spreading to most parts of the central nervous system (CNS) like the cerebrum, diencephalon, cerebellum, pons and medulla [5]. In addition, it was found that CA8 can be detected in different types of mouse tissues, with more intense expression in Purkinje cells [6]. Up to date, the only known biochemical function of CA8 is that it influences inositol 1,4,5-triphosphate (IP_3) binding to its receptor IP_3R1 on the endoplasmic reticulum and therefore modulates calcium signaling [6].

In 2005, a 19-bp deletion in exon 8 of the *CA8* gene (*ca8*) was found to cause ataxia and a lifelong gait disorder in waddles mice [7]. In addition, a homozygous mutation, S100P, in human *ca8* gene has been described in a consanguineous Iraqi family in which affected siblings had mild mental retardation and congenital ataxia characterized by quadrupedal gait [8]. Recently, the other homozygous mutation in CA8, G162R, was also identified in patients with variable cerebellar ataxia and mild cognitive impairment without quadrupedal gait [9]. These studies indicated that CA8 plays an important role in motor coordination and can be involved in human neurodegenerative disorders. On the other hand, significant expression of CA8 was observed in invasive lung adenocarcinoma but not in noninvasive adenocarcinoma. Interestingly, CA8 was strongly expressed in signet-ring cell cancer and invasive mucinous adenocarcinoma components [10]. Increased expression of CA8 has also been shown in colorectal carcinoma [11]. In addition, cell proliferation, colony formation, and cell invasion assays all demonstrated that colon cancer cell line (LoVo) bearing CA8 expression, LoVo-CA8, has

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http://dx.doi.org/10.1016/j.bbagen.2014.04.017
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significantly higher cell proliferative and invasive abilities than those found in parental LoVo and control LoVo-pCIneo cells in vitro. Furthermore, in vivo xenograft assay showed a higher tumor growth rate in LoVo-CA8 cells than that in parental LoVo cells [11]. Taken together, down-regulated CA8 and CA8 mutation may cause deficiencies in motor control but overexpression of CA8 may promote cell oncogenesis. However, the mechanisms that CA8 may be involved in the neural function and the exact biological functions of CA8 are still not clear.

In this study, we examined the functions of CA8 in neuronal cells because CA8 is distinctly expressed in the neural cells. First, the endogenous expression of CA8 was assessed in three easily accessible neuronal cell lines, including IMR32, SK-N-SH and Neuro-2a cells, as well as in mouse cerebellar granule neurons. To our surprise, no detectable expression of CA8 proteins was observed in neuroblastoma cell lines. Therefore, CA8 overexpression stable clones were established in SK-N-SH and Neuro-2a cells for analyzing the effects of CA8 overexpression. On the other hand, to examine the results of abolishing endogenous CA8 expression in normal neural cells, CA8 shRNA was used to eliminate the endogenous CA8 expression in cerebellar granule neurons. Together by using cell proliferation, cell migration/invasion, and staurosporine (STS)-induced cell apoptosis assays, we presented the results to define the functions of CA8 in neuron cells. Furthermore, to evaluate whether zebrafish may be used as an animal model to study the functions of CA8, we used *ca8* morpholino (*ca8* MO) to down-regulate CA8 expression for the observation of morphological and behavior changes in zebrafish, as well as the expression patterns of CA8 during embryo development.

2. Materials and methods

2.1. Reagents and antibodies

Human neuroblastoma cell line SK-N-SH (HTB-11; ATCC) was provided by Dr. Shin-Lan Hsu (Taichung Veterans General Hospital, Taiwan); human neuroblastoma cell line IMR32 (CCL-127; ATCC) and mouse neuroblastoma cell line Neuro-2a (CCL-131; ATCC) were purchased from Bioresource Collection and Research Center (Hsin-Chu, Taiwan). All materials for cell culture were purchased from Gibco Life Technologies (Gaithersburg, MD). Reagents for Western blot were obtained from Pierce (Rockford, USA). Rabbit polyclonal antibody specific for CA8 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse monoclonal antibody specific for Parvalbumin7 was provided by Dr. Masahiko Hibi (Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan); mouse monoclonal anti-α-tubulin was purchased from Sigma (St. Louis, MO, USA); rabbit polyclonal anti-IP_3R1 was purchased from Millipore (Billerica, MA, USA). To generate plasmid pLKOAS3w-CA8.neo, a lentiviral transfer vector containing the full length CA8, CA8 DNA fragment was amplified by PCR and inserted between *Nhe*I and *Pme*I of pLKOAS3w.neo; plasmid pLKOAS3w-CA8-myc.puro, a lentiviral transfer vector containing the full length CA8 with myc tagged, was generated by PCR amplifying CA8-myc DNA fragment and inserted between *Nhe*I and *EcoR*I of pLKOAS3w.puro. The recombinant lentiviral production was generated by the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Meanwhile, control virus and virus carrying CA8 RNA interference (RNAi) vector, pLKO.1-shCA8 (shCA8-1, TRCN000153276; shCA8-2, TRCN000155916), were obtained from the National RNAi Core Facility.

2.2. Cell cultures

SK-N-SH, IMR-32 and Neuro-2a cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL pyruvate, 1% PS, and 1% non-essential amino acid, and maintained at 37 °C in a humidified atmosphere with 5% CO_2. SK-N-SH cells were sub-cultivated every three days at a ratio of 1:2; IMR-32 cells were sub-cultivated every three days at a ratio of 1:3 and Neuro-2a cells were sub-cultivated every three days at a ratio of 1:6.

2.3. Plasmid construction

Full-length CA8 cDNA was PCR amplified from pcDNA3.1-CA8-myc-His (Chang W.H., unpublished work) using synthesized primers, CA8-*Nhe*I (5′TCCTGATGCTAATGGGGAATACCAG 3′) and CA8-*Pme*I (5′CTAAGAGGCTGAGTGGGCCGAAAG 3′). CA8 cDNA was then subcloned into pLKOAS3w.neo lentiviral vector and their DNA sequences were confirmed.

2.4. Selection of stable cells and Western blot analysis

The recombinant pLKOAS3w-CA8.neo lentiviral production was generated by the National RNAi Core Facility (Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan). Infection of this modified virus for stable expression of CA8 was performed according to the instructions provided by the National RNAi Core Facility. SK-N-SH and Neuro-2a cells were infected with recombinant lentivirus and lines were then selected using 500 μg/ml of G418 (GIBCO/BRL). Expression of CA8 proteins was examined by Western blot analysis using anti-CA8. After seven days of selection, the survival cells were diluted to a concentration of 10 cells/ml and keep growing in the medium containing 500 μg/ml of G418. Stable cells were collected after 14–20 days. Western blot analysis was performed as previously described [12].

2.5. Cell toxicity studies and cell survival analysis

SK-N-SH, SK-N-SH-CA8, Neuro-2a and Neuro-2a-CA8 cells were maintained in DMEM containing 10% fetal bovine serum and then 2×10^5 (Neuro-2a and Neuro-2a-CA8) and 4×10^5 (SK-N-SH and SK-N-SH-CA8) cells were seeded in 3 cm dishes. One day after seeding, cultured medium was replaced with a medium containing 500 nM (Neuro-2a) and 1 μM (SK-N-SH) STS and cells were incubated in this medium for different periods at 37 °C.

2.6. Characterization of apoptotic cells

Control or STS-treated cells were harvested by trypsin incubation, washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS buffer for 30 min at room temperature (RT), washed three times with PBST (0.1% of Tween 20 in PBS) and then exposed to 20 μg/ml of propidium iodide (PI) in PBS for 15 min at RT, and washed three times with PBST. Apoptotic cells exhibited highly fluorescent condensed or fragmented DNA by fluorescence microscopy.

2.7. Transwell cell migration and invasion assays

Cell migration was studied using inserts incorporating 8 μm polyethylene terephthalate (PET) track-etched membranes transwell coated with 10 μg/ml collagen IV as chemo-attractant. Trypsinized cells were suspended in serum free medium and 1×10^5 cells were seeded to the upper chamber of transwell inserts. 3% fetal bovine serum medium was added to the lower chamber. After 12 h of incubation for SK-N-SH and primary granule neurons, non-migrated cells on the upper surface of the membrane were scrapped off, cells on the lower surface were stained with 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) in PBS and counted from 7 to 8 randomly selected fields. Cell invasion assay was performed using matrigel coated (50 μl of 5% matrigel) transwell inserts as described previously [13]. Invasion assay was performed similar to migration assay, only the cells were incubated for 36 h before observation. All the data were obtained from three separate experiments.

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