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Expressional and functional studies of Wolframin, the gene function deficient in Wolfram syndrome, in mice and patient cells

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Abstract

Wolfram Syndrome is an autosomal recessive degenerative disorder of the neuroendocrine system. Diabetes mellitus is its lead symptom. Patients show mutations in the wolframin (WFS1) gene coding for a hydrophobic transmembrane protein of 890 amino acids. This protein was preliminarily localised in the endoplasmatic reticulum (ER) in cells of mice and rats. Mice lacking the WFS1 gene display degeneration of pancreatic β-cells following induction of ER stress.

We here used antibodies against substructures of the wolframin protein in order to analyse its expression and localisation. Expression was detected in both pancreatic β-cells and the limbic system of mice. Using the rat insulinoma cell line RIN 5AH and fractionated mouse brain tissue, we confirmed wolframin localisation to the endoplasmic reticulum.

Expression profiling on patient's primary fibroblasts revealed down-regulation of the diabetes associated plasma membrane glycoprotein (PC-1) gene, and up-regulation of fibulin-3, a gene connected to senescence. However, cell proliferation was indistinguishable from non-mutated cells. In contrast to data obtained on murine pancreatic islets, we found no increased apoptosis following induction of ER stress but rather by staurosporine treatment in the absence of WFS1 function. This indicates a new role of WFS1 deficiency in programmed cell death.

Keywords: DIDMOAD; Wolframin; Endoplasmatic reticulum; Apoptosis; β-cells

1. Introduction

Wolfram or DIDMOAD Syndrome is clinically characterised by diabetes mellitus, diabetes insipidus, optical atrophy and deafness (Fraser and Gunn, 1977; Richardson and Hamilton, 1977). Generally, diabetes mellitus appears in the first decade of life and optical atrophy in the second, while the other symptoms are not obligatory (Barrett and Bundey, 1997). In addition, other neurological features such as ataxia, nystagmus, peripheral neuropathies and mental retardation may appear (Barrett et al., 1995). Occasionally, pituitary-gonadal malfunctions are observed (Peden et al., 1986) and predisposition to psychiatric illness has been associated with this disease (Evans et al., 2000). In general, Wolfram syndrome patients die at 25–40 years of age from central respiratory failure, presumably caused by brainstem atrophy (Barrett et al., 1995). The variety and multitude of symptoms are consistent with the disease representing a progressive neurodegenerative disorder affecting the central and peripheral nervous systems (Barrett and Bundey, 1997).

Wolfram syndrome is an autosomal recessive disorder due to mutations on both alleles of a single gene located on chromosome 4p16.1, WFS1, encoding wolframin (Inoue et al., 1998; Strom et al., 1998). Interestingly, heterozygous mutations in the WFS1 gene are associated with the dominantly inherited disease, Low Frequency Sensorineural Hearing Loss (LFSNHL) (Bespalova et al., 2001; Lesperance and Hall, 2003).

mRNA analyses of the WFS1 gene revealed expression in a variety of tissues (Inoue et al., 1998; Strom et al., 1998). The gene codes for a membrane protein of 890 amino acids, representing a molecular weight of about 100 kd. This hydrophobic macromolecule containing nine hypothetical

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membrane passages, was found to be a glycoprotein and localized to the endoplasmic reticulum (ER) in transfected COS-7 cells (Takeda et al., 2001), in the pancreatic β-cell line, BRIN-BD11 (Osman et al., 2003), and in several cell types within the inner ear (Cryns et al., 2003). Ishihara et al. have recently shown that disruption of the WFS1 gene in mice caused progressive β-cell loss and impaired stimulus secretion coupling in insulin secretion (Ishihara et al., 2004).

In order to further study the localisation and function of the human wolframin protein, we generated antibodies to substructures of its open reading frame and used them in immunohistological and immunohistochemical studies. In addition, we analysed primary fibroblast cultures from Wolfram syndrome patients. Expanding previous observations, we find that endogenous wolframin is an ER resident membrane protein that is expressed in substructures of the brain and in pancreatic β-cells of the mouse. We also identified genes differentially expressed in fibroblast cells from a Wolfram syndrome patient. Finally, we show that reduced wolframin expression renders fibroblast cells hypersensitive to apoptotic stimuli others than those inducing endoplasmatic reticulum stress.

2. Materials and methods

2.1. Antibodies and cells

A Chou-Fasman plot prediction program was used to determine the areas most suitable for peptide synthesis. Four peptides were synthesized from the N-terminal region, conjugated to the Keyhole Limpet Hemocyanin carrier protein and used to immunize rabbits. Of these four peptides, only one, m82 (KLNPKKKKQV AVSEL-LENVG, amino acids 203–222) showed specific reactivity. A recombinant antibody generated against the N-terminal hydrophilic domain, anti-WoN, was kindly provided by S. Hofmann (Hofmann et al., 2003). Anti-PDI (protein disulfide isomerase) mAb and anti-58k (Golgi) were purchased from Sigma. Anti-Calnexin was purchased from Chemicon and Mitotracker was from Molecular Probes. Human skin fibroblasts and the rat insulinoma cell line, RIN 5AH were grown in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax supplemented with 10% fetal calf serum (FCS). The insulinoma cell lines, βTC-tet (mouse) and R7T1 (rat) were grown in DMEM plus Glutamax supplemented with 2.5% FCS and 15% horse serum and R7T1 was additionally supplemented with tetracycline for propagation. The rat α-cell line, In-RI-G9 was grown in RPMI medium supplemented with 10% FCS.

2.2. Cell extraction and sub-cellular fractionation

Crude cellular extracts from cultured fibroblasts were prepared by directly boiling cell pellets at a concentration of 1×10^4 cells/μl in 1× Laemmli buffer (60 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and bromophenol blue) for 5 min at 95 °C.

Mouse brain tissue was isolated and kept in ice-cold HES buffer (10 mM HEPES, 250 mM sucrose, 1 mM EDTA and 1 mM PMSF). Homogenisation was done with 6 strokes of a glass/teflon potter and 2 strokes in a glass douncer with a loose fitting and once with a tight fitting. This homogenate was centrifuged at 600×g for 10 min. to pellet out cell debris. The supernatant was centrifuged 3 times at 1,000×g for 10 min each to ensure that no other nuclear debris would contaminate the mitochondrial fraction. Pellets were pooled from the second and third centrifugation and used as the nuclear pellet. The supernatant was first centrifuged at 8,000×g for 10 min and the resulting pellet was then washed in 5 ml HES buffer and centrifuged at 10,000×g for 10 min. The mitochondrial fraction was created by dissolving the pellet in 2 ml HES buffer and purifying over a sucrose gradient of 1.2 and 1.5 M sucrose by ultracentrifugation at 40,000×g for 25 min. The 10,000×g post-mitochondrial supernatant was centrifuged at 100,000×g for 1 h to separate the microsomal fraction (pellet) from the cytosol fraction (supernatant). Protein concentrations were measured using Roti-Quant (Roth).

2.3. Western blot analysis

Protein extractions were separated on 10% polyacrylamide gels and electroblotted onto 0.2 μm nitrocellulose membranes. 5% non-fat dried milk dissolved in Tris-buffered saline solution containing 0.1% Tween-20 was used to block the membranes. Primary antibodies were allowed to incubate overnight at 4 °C, after which the secondary horseradish peroxidase-conjucated anti-rabbit or anti-mouse (Sigma) was added. Detection was performed using the enhanced chemiluminescence (ECL, Amersham) system.

2.4. Immunofluorecence

Cells were seeded onto 8-well chamber slides (Nunc) to approximately 80% confluency. Slides were then washed in PBS and fixed for 10 min at room temperature with 4% Paraformaldehyde in PBS and permeabilized using Triton X-100. Primary antibodies were diluted in PBS containing 3% bovine serum albumin (BSA) and 0.2% Triton X-100 and incubated for 1 h at room temperature. Either a FITC or TRITC conjugated secondary antibody (Sigma) was added at a dilution of 1:50 for 20–30 min at room temperature.

For tissues, 5 μm thick mouse pancreas cryosections were fixed for 10 min in acetone. The sections were then blocked for 30 min at room temperature with normal goat serum at a 1:50 dilution in PBS. Anti–WoN antibody was diluted to a concentration of 1:1000 in PBS containing 0.5% goat serum and incubated overnight at 4 °C in a humid chamber. Anti-insulin (Biogenesis) and anti-glucagon (Sigma) were used at a dilution of 1:1000. FITC or