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Cerebral ischemic injury is enhanced in a model of oculodentodigital dysplasia

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ABSTRACT

Oculodentodigital dysplasia (ODDD) is a rare autosomal dominant disease that results in visible developmental anomalies of the limbs, face, eyes and teeth. Recently analysis of human connexin43 (Cx43) DNA sequences has revealed a number of different missense, duplication and frame shift mutations resulting in this phenotype. A mouse model of this disorder has been created with a missense point mutation of the glycine amino acid at position 60 to serine (G60S). Heterozygote +/G60S mice exhibit a similar ODDD phenotype as observed in humans. In addition to the malformations listed above, ODDD patients often have neurological findings. In the brain, Cx43 is highly expressed in astrocytes and has been shown to play a role in neuroprotection. We were interested in determining the effect of the +/G60S mutation following stroke. Four days after middle cerebral artery occlusion the volume of infarct was larger in mice with the +/G60S mutation. In astrocyte-neuron co-cultures, exposure to glutamate also resulted in greater cellular death in the +/G60S mutants. Protein levels of Cx43 in the mutant mouse were found to be reduced when compared to the normal tissue. Cx43 protein was observed as a continual line of small punctate aggregates in the plasma membrane with increased intracellular localization, which is distinct from the larger plaques seen in the normal mouse astrocytes. Functionally, primary +/G60S astrocytes exhibited reduced gap junctional coupling and increased hemichannel activity, which may underlie the mechanism of increased damage during stroke.

This article is part of the Special Issue Section entitled 'Current Pharmacology of Gap Junction Channels and Hemichannels'.

1. Introduction

Oculodentodigital dysplasia (ODDD) was first described by Meyer-Schwickerath et al. (1957) and further defined by Gorlin et al. (1963). Common external features in this syndrome include ocular, nasal, dental and digital abnormalities (Paznekas et al., 2009). It is now known that this syndrome is caused by mutation of the gene encoding the gap junction protein connexin43 (Cx43). Currently, 62 different Cx43 mutations have been identified in ODDD patients, and in the majority of cases the mutation is dominant (Paznekas et al., 2009).

Cx43 is expressed in numerous tissues and plays an important role in cellular communication. Cx43 proteins oligomerize into hexamers to form connexons, which are inserted into the plasma membrane of single cells to form hemichannels, or coupled to the connexons in neighboring cells to form gap junction channels that provide cytoplasmic continuity between cells (Sáez et al., 2003). The effect of ODDD Cx43 mutations has been studied by several groups and in general these mutants show reduced gap junction formation and increased hemichannel activity (Dobrowolski et al., 2007; McLachlan et al., 2005). Also, mutated Cx43 often exerts a

Abbreviations: BSA, bovine serum albumin; Cx43, connexin43; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; Etd, ethidium; PI, propidium iodide; La^{3+}, lanthanum ion; G60S, missense point mutation of the glycine amino acid at position 60 conversion to serine; GFAP, glial fibrillary acidic protein; HBSS, Hank's buffered salt solution; IBA-1, ionized calcium-binding adaptor molecule 1; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; ODDD, oculodentodigital dysplasia; RIPA, radioimmune precipitation lysis buffer; TBS-T, Tris-buffered saline with tween.

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dominant-negative effect by reducing normal Cx43 function (McLachlan et al., 2005). However, recent studies have suggested that gap junction coupling and hemichannel activity are not affected in G60S ODDD astrocytes (Wasseff et al., 2011) and C6 glioma cells expressing some ODDD mutations (Gutmann et al., 1991; Lai et al., 2006).

In the brain, Cx43 is highly expressed in astrocytes, and understanding the effect of the ODDD Cx43 mutations on astrocytes remains to be clarified. There is reason to believe that mutations of Cx43 have an effect on the nervous system as ODDD patients often have neurological symptoms including ataxia, dysarthria, neurogenic bladder, seizures and spastic paraparesis (Flenniken et al., 2005; Gutmann et al., 1991; Loddenkemper et al., 2002). We have previously shown that disruption of Cx43 leads to increased infarct volume following middle cerebral artery occlusion (MCAO) (Kozoriz et al., 2010; Nakase et al., 2004; Siushansian et al., 2001), however the effect of an ODDD mutation in ischemia is not known. In this study we were interested in determining if cellular susceptibility to stroke is altered in a mouse with a +/G60S point mutation that exhibits an ODDD phenotype (Flenniken et al., 2005). We examined infarct volume following MCAO and neuronal death in a neuron-astrocyte co-culture preparation. We also assessed the pattern of astrocytic Cx43 expression, gap junction coupling and hemichannel activity.

Taken together, the results of our study indicate that the +/G60S Cx43 mutation results in reduced astrocytic gap junctional communication and enhanced hemichannel activity; both of these could contribute to impaired neuroprotection.

2. Material and methods

All experiments were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by The University of British Columbia Animal Care Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

2.1. ODDD mice and MCAO

Mice from a C57BL/6 genetic background were bred with heterozygote mice harboring a +/G60S point mutation ($Gja^{Jrt}/+$ strain) (Flenniken et al., 2005). The mice used in this study were obtained from the Centre for Modeling Human Disease, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto Centre for Phenogenomics, Toronto, ON Canada. They were on a FVB/NJ (25%), C3H/HEJ (25%) and C57BL/6 (50%) genetic background. In this manuscript, wild-type refers to $Cx43^{+/+}$ mice while +/G60S refers to the autosomal dominant $Cx43^{Jrt/+}$ mutant. Genotyping for endogenous and +/G60S Cx43 has been previously described (Flenniken et al., 2005; Naus et al., 1997). The MCAO surgery was performed as previously described by our lab (Nakase et al., 2003, 2004; Siushansian et al., 2001). In brief, mice aged 8–10 weeks were anaesthetized with sodium pentobarbital (65 mg/kg intraperitoneally; MTC Pharmaceuticals, Cambridge, Canada). The mice were secured to a 37 °C heating pad in a sterotaxic frame, a skin incision was made and the squamosal bone was exposed by retracting the temporalis muscle. A burr hole was made over the middle cerebral artery and the dura mater was pierced with a needle. The MCA was then occluded above and below the rhinal fissure by electrocautery. The skin incision was closed with sutures and the mice were kept in a cage warmed on a 37 °C heating pad until regaining consciousness.

2.2. Infarct volume

For this study a total of four mice for each genotype were used. Infarct volume was assessed as described in our previous studies (Kozoriz et al., 2010; Nakase et al., 2004; Siushansian et al., 2001). Four days after MCAO mice were deeply anesthetized with sodium pentobarbital (70 mg/kg intraperitoneally) and were transcardially perfused with phosphate-buffered saline (PBS) followed by perfusion with 10% formalin (Sigma–Aldrich, Oakville, Canada). Brains were removed and stored in 10% formalin and the next day were cryoprotected in a 10% formalin/30% sucrose solution. Brain sections, 20 μm thick, were collected every 100 μm, and mounted sequentially on glass microscope slides. To quantify infarct size sections were stained with 0.125% thionin (Fisher Scientific, Ottawa, Canada) and the volume of infarct was measured by adding together the lesion area in each of the serial sections.

2.3. Astrocyte cultures

Astrocyte cultures from both wild-type and ODDD mice were prepared from 0 to 1 day old pups (Kozoriz et al., 2010; Ozog et al., 2002). Neocortices were dissected in PBS and placed in culture medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Burlington, Canada) with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 10 U/mL penicillin, and 10 U/mL streptomycin (Invitrogen Corp.). Tissue was triturated through a serological pipette and passed through a 70 μm cell strainer (BD Falcon, Bedford, MA). Cells were re-suspended in culture medium and plated on either plastic culture dishes or laminin coated coverslips and stored in a humidified incubator in 95% air/5% CO_2 at 37 °C. Medium changes were performed every 4 days and astrocytes were used between 14 and 21 days *in vitro* or when 30% confluent for the hemichannel experiments described below (~5 days).

2.4. Neuron-astrocyte co-culture cytotoxicity assay

Neuron-astrocyte co-cultures were generated as previously described (Ozog et al., 2002). In brief, cultured astrocytes, prepared as described above, from each genotype were grown until confluent (10–13 days) in 35 mm plastic culture dishes. Wild-type neurons (1×10^6 cells/well) isolated from gestational age day 16 mice were seeded on top of the astrocyte monolayer. The co-culture media contained 3/5 neurobasal medium, 2/5 DMEM/F12, supplemented with 10 U/mL penicillin, 10 U/mL streptomycin and B27 supplements (Invitrogen Corp.). Media changes were performed every 3 days by replacing 2/3 of the solution with fresh co-culture media.

On day 8, co-cultured cells was rinsed with Earle's balanced salt solution (EBSS) and exposed to 1 mM glutamate (Sigma–Aldrich, St. Louis, MO) or vehicle in EBSS for 3 h. Cells were then maintained in co-culture media for 24 h after glutamate/vehicle exposure. Next, samples of media were collected and frozen for subsequent analysis of lactate dehydrogenase (LDH) release using an LDH detection kit (Sigma–Aldrich). Cell cultures were also exposed to propidium iodide (30 μM in PBS) for 5 min and subsequently fixed in 4% formaldehyde solution. The tissue was mounted with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes Inc., Eugene, OR).

2.5. Immunohistochemistry

Immunohistochemistry was performed on cortical tissue or on cultured astrocytes from each genotype as previously described (Kozoriz et al., 2010; Nakase et al., 2004; Ozog et al., 2002). Ten μm thick glass mounted sections were rinsed in PBS and then exposed to 0.3% Triton-X-100 (Fisher Scientific) in PBS for 2 min and then rinsed in PBS. Sections were blocked in 7.5% bovine serum albumin (BSA; Sigma–Aldrich) for 30 min, incubated overnight in 1% BSA–PBS and primary antibody, then washed with PBS (3 × 10 min) and subsequently incubated for 1 h with secondary antibody in 1% BSA–PBS. Slides were then rinsed 3 times for 10 min with PBS, dipped in water, and mounted with ProLong Gold antifade reagent with DAPI. Primary antibodies were used at the concentrations indicated: anti-Cx43 C-terminal (amino acid residues 363–382) antibody raised in rabbit (1:2000 dilution; Sigma–Aldrich; catalog #C6219), anti-glial fibrillary acidic protein (GFAP) antibody raised in mouse (1:1000; Sigma–Aldrich; catalog #G3893) and anti-ionized calcium-binding adaptor molecule 1 (IBA-1) antibody produced in rabbit (1:500; Wako Pure Chemical Industries, Richmond, VA; catalog #019-19741). Secondary antibodies consisted of a 1:500 dilution of highly cross-adsorbed goat anti-rabbit or anti-mouse IgG antibodies conjugated to Alexa Fluor 488 (catalog #A-11029 and #A-11034) or Alexa Fluor 568 (catalog #A-11031 and #A-11036) and were used as appropriate (Molecular Probes Inc.). Images were obtained using the same exposure times on a Leica TCS SP5 II Basic VIS system (Leica Microsystems Canada Inc., Concord, Ontario, Canada). Identical conditions were used for acquisition of immunofluorescence images.

Immunocytochemistry on confluent astrocyte cultures was performed using the same concentration of Cx43 antibodies and secondary antibodies as used on tissue sections noted above. Astrocytes were rinsed in PBS, fixed in 4% paraformaldehyde, rinsed in PBS and then exposed to 0.1% Triton-X-100 PBS (Fisher Scientific, Ottawa, Canada) for 2 min. After a brief PBS rinse, cells were blocked in 7.5% BSA for 30 min. Astrocytes were then incubated for 1 h in PBS containing 1% BSA and primary antibody, then washed with PBS 3 times for 10 min and incubated for 1 h with a secondary antibody in PBS containing 1% BSA. Astrocytes were then rinsed 3 times for 10 min with PBS and mounted with ProLong Gold with DAPI. Images were obtained using the same exposure times on a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss Ltd, Toronto, Canada). Identical conditions were used for acquisition of immunofluorescence images.

2.6. Western blot

Adult cortical brain tissue and astrocyte cultures were prepared and probed using standard Western blot protocols (Siushansian et al., 2001). In brief, tissue was lysed in radioimmune precipitation lysis buffer (RIPA) supplemented with protease inhibitors (Roche Laval, QC, Canada) and phosphatase inhibitors (Sigma–Aldrich). Samples were extracted with a syringe through a 26 gauge needle,