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Connexin43 confers Temozolomide resistance in human glioma cells by modulating the mitochondrial apoptosis pathway

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive astrocytoma, and therapeutic options are generally limited to surgical resection, radiotherapy, and Temozolomide (TMZ) chemotherapy. TMZ is a DNA alkylating agent that causes DNA damage and induces cell death. Unfortunately, glioma cells often develop resistance to TMZ treatment, with DNA de-methylation of the MGMT promoter identified as the primary reason. However, the contributions from proteins that normally protect cells against cytotoxic stress in TMZ-induced apoptosis have not been extensively explored. Here, we showed that increasing the level of the gap junction protein, Cx43, in human LN18 and LN229 glioma cells enhances resistance to TMZ treatment while knockdown of Cx43 in these same cells sensitizes them to TMZ treatment. By expressing a channel-dead or a C-terminal truncation mutant of Cx43, we show that Cx43-mediated TMZ resistance involves both channel dependent and independent functions. Expression of Cx43 in LN229 cells decreases TMZ-induced apoptosis, as determined by Annexin V staining. Cx43-mediated chemoresistance appears to be acting via a mitochondrial apoptosis pathway as manifested by the reduction in Bax/Bcl-2 ratio and the release of cytochrome C. Our findings highlight additional mechanisms and proteins that contribute to TMZ resistance, and raise the possibility of increasing TMZ efficiency by targeting Cx43 protein.

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1. Introduction

Gliomas are the most common neoplasm of the central nervous system. The most aggressive subtype is glioblastoma multiforme (GBM), a World Health Organization grade IV astrocytoma that is associated with very poor prognosis, with only 3–5% of patients surviving more than 3 years (Krex et al., 2007). With a median survival of approximately 14 months, GBM is considered the most lethal type of primary brain tumors in adults (Stupp et al., 2005). GBM patients generally receive a multimodal approach of surgical resection, radiotherapy and chemotherapy using the DNA alkylating agent Temozolomide (TMZ). Although neurosurgery can remove more than 90% of a tumor mass (Hentschel and Sawaya, 2003; Lesniak et al., 2003), cells migrating away from the core often establish secondary tumors that are accountable for high GBM mortality. TMZ is currently the most effective chemotherapeutic demonstrating excellent CNS bioavailability (Neyns et al., 2010; Stupp et al., 2005), causing DNA methyl adducts on the N^7 of guanine, N^3 of adenine and O^6 position of guanine, about 70%, 9% and 5% respectively (Villano et al., 2009). The latter DNA adduct is primarily responsible for the cytotoxic effect, however, clinical response toward TMZ is generally short-lived as tumor cell heterogeneity, transformation, genetic instability and selective pressures (Salvati et al., 2009) contribute to the development of chemoresistant tumors (Giese et al., 2003). O-6-methylguanine-DNA methyltransferase (MGMT) can remove toxic TMZ-induced DNA methylation and its expression is correlated to poor TMZ response (Esteller et al., 2000). More recently, a role was found for the mismatch repair (MMR) pathway and the base excision repair (BER) pathway in TMZ resistance (Sarkaria et al., 2008; Yip et al., 2009).

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Additional pathways, such as activation of DNA damage checkpoint response and the changes in mitochondria DNA and electron transport chain may contribute to TMZ resistance (Frosina, 2009; Oliva et al., 2010). In fact, chemoresistance in gliomas is associated with reduction of reactive oxygen species (ROS) and the activity of cytochrome C oxidase (Oliva et al., 2011). In this way, TMZ reduces the viability of glioblastoma cells by triggering apoptosis, which is accompanied by increased loss of mitochondrial-membrane potential, cytochrome C (Cyt C) release, caspase activation and caspase-dependent apoptosis (Wagner et al., 2013).

The connexins are tetra-span integral plasma membrane proteins, comprised of two extracellular loops and one intracellular loop along with cytoplasmic amino (NH_2) and carboxyl-terminal (CT) tail domains. Required for a broad range of physiological processes (Naus et al., 1992; Sabarinathan et al., 2010), gap junctions (GJs) form intercellular ion channel arrays that permit the passage of small ions and molecules to pass between neighboring cells (Fry et al., 2001; Goldberg et al., 1999; Simon and Goodenough, 1998). There are two mutually inclusive mechanisms that can explain the regulatory functions linked to connexins. The most traditional view, first noted by Loewenstein and Kann, acknowledges their role as channels involved with direct cell–cell communication that is frequently lost or reduced in cancer cells (Loewenstein and Kanno, 1966). Connexins facilitate communication between the cytosol and the extracellular space through GJ "hemichannels" located at the plasma membrane surface (Goodenough and Paul, 2003). Such channel-forming roles could include the transmission of factors that suppress growth and dispersion of tumor promoting agents with neighboring cells and/or extracellular space. In the second mechanism, connexins execute their tumor suppressor properties through channel-independent pathways (Goodenough and Paul, 2003; Naus and Laird, 2010). For example, the GJ protein connexin43 (Cx43) has been shown to reverse the oncogenicity of human glioma cells in the absence of gap junctional intercellular communication (GJIC) (Huang et al., 1998). The non-channel mechanism of action likely involve the protein's long cytoplasmic CT tail (Giepmans, 2004; Herve et al., 2004) that not only serves as a platform for protein–protein interaction and supermolecular assembly, but regulation by post-translational modification and proteolytic cleavage (Chen et al., 2012).

Various studies in gliomas have demonstrated a role of Cx43 in glioma growth control and migration [reviewed in (Sin et al., 2012)]. The reduction of GJIC in tumor cells is often attributed to reduced Cx43 expression (Mesnil et al., 2005). Therefore, introduction of oncogenes and mitogens to cultured cells interfere with GJIC by reducing the number of connexin-containing GJ plaques and/or alter its trafficking to the plasma membrane (Laird et al., 1995; Naus and Laird, 2010). The role of Cx43 in apoptosis is less well studied and it remains controversial whether GJIC is pro- or anti-apoptotic (Sin et al., 2012). Recent evidence suggests the hemichannel activity of Cx43 may have a more significant role than intercellular channel activity in affecting cell death (De Vuyst et al., 2009; Decrock et al., 2009; Retamal et al., 2006), and identification of Cx43 in mitochondria suggests GJ proteins may influence pathways linked to apoptosis control (Lu et al., 2010; Ruiz-Meana et al., 2008). Here, we show for the first time that Cx43 expression is linked to TMZ-acquired resistance in human glioma cells. Using a Cx43 channel defective (T154A) mutant and a CT truncated Cx43 mutant terminated at amino acid 243 (TrCx43), we demonstrate that Cx43-dependent TMZ resistance depends on both its channel properties and channel-independent mechanisms. We further show that Cx43 alters mitochondrial apoptotic pathways by regulating levels of Bax2 and Bcl-2, as well as Cyt C release from the mitochondria following TMZ treatment.

2. Materials and methods

2.1. Reagents and antibodies

Rabbit polyclonal anti-Cx43 (Sigma, St Louis, USA C6219), mouse monoclonal anti-GAPDH (HyTest Ltd, mAb 6C5), rabbit anti Bcl-2 (Santa Cruz), rabbit anti-Bax (Cell Signaling), HRPO-conjugated donkey anti-rabbit/mouse IgG (Sigma) and anti-cytochrome C (Santa Cruz, clone 7H8) were used in this study. TMZ (≥98% purity, T2577) was obtained from Sigma. Unless otherwise stated, all other reagents and buffers were obtained at biotechnology grade from ThermoFisher Scientific (Nepean, ON, Canada).

2.2. Immunohistochemistry

Human brain tumor and normal tissue microarray slides (GL2083 from US Biomax) containing 208 tissue cores were processed by Wax-it Histology Services Inc. (Vancouver, Canada). Briefly, formalin-fixed paraffin embedded tissue sections of 1 mm core diameter and 5 μm thick were deparaffinized in xylene and rehydrated in graded alcohols. Sections were boiled in sodium citrate buffer, pH 6.0, for 15 min for antigen retrieval, then treated with 3% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity, and blocked with protein block (DAKO) to reduce background staining. Sections were then incubated with anti-Cx43 antibody (Sigma) overnight at 4 °C. The following day, the sections were incubated with the Envision + System-Horse Radish Peroxidase Labeled Polymer. Cx43 immunoreactivity was visualized with diaminobenzidine (DAB). Sections were counterstained in Mayer's hematoxylin, blued in lithium carbonate, dehydrated in graded alcohols, cleared in xylene, and mounted with permount (Fisher Scientific). Slides were visualized with Aperio ImageScope of the ScanScope digital slide scanner.

2.3. Cell culture and generation of stable cell lines

Human LN229 glioma cells (ATCC, Manassas, VA, USA) were infected with retroviral pMSCVpuro vectors with either wild-type Cx43 (designated as Cx43), Cx43 truncated at amino acid 243 (TrCx43), or with an empty plasmid (Mock) as described previously (Crespin et al., 2010). The channel dead Cx43 mutant (T154A) (Beahm et al., 2006) was generated using a site directed mutagenesis kit (Qiagen) with the primer pair (forward: 5′ GGCTTGCTGAGAGCCTACATCATCAGCATCC 3′; reverse complement: 5′ GGATGCTGATGATGTAGGCTCTCAGCAAGCC 3′) and inserted into a pMSCV vector (Clontech). Cells were cultured in media consisting of high glucose DMEM (Sigma, St Louis, USA) supplemented with 5% (v/v) fetal bovine serum (HyClone/ThermoFisher Scientific), puromycin (2 μg/ml), 1% (100 μg/ml) penicillin–streptomycin (10,000 μg/ml, Invitrogen, Burlington, On, Canada). Human LN18 glioma cells (ATCC) were cultured in low glucose DMEM (Sigma) supplemented with 10% (v/v) fetal bovine serum and puromycin (800 mg/ml) as previously described (Crespin et al., 2010). All cells were incubated at 37 °C in a 5% CO_2 atmosphere (Thermo Forma, model 310). Cells were passaged every three days after washing with PBS (50 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl solution) using trypsin–0.25% EDTA (Invitrogen). For shRNA knockdown experiments, LN229-Cx43 cells were treated with two rounds of infection with retrovirus containing a scrambled sequence and a Cx43-shRNA sequence as reported by Bates et al. (Bates et al., 2007). After 48 h, Cx43-shRNA cells were selected in the presence of 40 μg/ml hygromycin.

2.4. Dye coupling assay

Evaluation of GJIC was examined as previously described (Bates et al., 2007) with some modifications. LN229 control and mutant cell lines were grown to confluency. Donor cells were treated with a solution of Calcein AM (Molecular Probes) and DiI (Sigma) and incubated for 20 min. The donor cells were then loaded onto another confluent plate of recipient cells at 1:100 ratio, and incubated for 2 h at 37 °C. The plates were rinsed with DMEM media to remove unattached donor cells. The cells were visualized under epifluorescence microscope to quantify coupling between cells.

2.5. Cytotoxic assay

TMZ was dissolved in DMSO to 0.1648 M as a stock solution and stored in aliquots at −20 °C prior to use. For the cytotoxic assay, cells were plated in a 96-well plate (Costar, Corning, USA) at 5000 cells per well in medium as described above. After 24 h, various concentrations of TMZ dispersed in medium were added to the cells. Control cells were treated with the corresponding volume of DMSO. After 72 h, the cells were washed twice with PBS and fixed for 10 min with 10% buffered formalin phosphate. Cells were stained with 0.05% crystal violet for 30 min. Excess stain was removed by washing with water. Crystal Violet was solubilized in isopropanol with 0.1% Igepal CA-630 and 0.033% HCl. Absorbance was measured after 10 min firm shaking at 562 nm on an EL_x808 Ultra microplate reader (Bio-Tek, Ottawa, Canada) for data analysis by two-way ANOVA.

For Cx43 phosphorylation studies, cells were plated overnight in a 6-well plate (BD Falcon, Franklin lakes, USA) at 250,000 cells per well in cell culture media. The