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QUERCETIN AND LITHIUM CHLORIDE MODULATE WNT SIGNALING IN PLURIPOTENT EMBRYONAL CARCINOMA NT2/D1 CELLS

MARIJA MOJSIN*, VLADANKA TOPALOVIĆ, JELENA MARJANOVIĆ and MILENA STEVANOVIĆ

Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11010 Belgrade, Serbia

Abstract - Wnt signaling functions in numerous cellular activities such as cell fate determination, patterning, and migration in embryogenesis, apoptosis, etc. In this study, we used quercetin and lithium chloride to investigate modulations of the Wnt signaling pathway in human pluripotent embryonal carcinoma NT2/D1 cell line. First, we optimized conditions for NT2/D1 cell treatments with quercetin and lithium chloride and assessed their cytotoxic effects on the cells, cell viability and proliferation rate. Our results showed that induction of cell death by quercetin and LiCl is p53-dependent in NT2/D cells. We also examined the degree of Wnt signaling modulations by analyzing the expression of *c-myc*, a well-known Wnt signaling target gene. Since the retinoic acid induction of NT2/D1 cells is good in an *in vitro* model system for human neural differentiation, studying Wnt signaling modulation in NT2/D1 would contribute to a better understanding of the mechanisms involved in neural stem cell maintenance and human neural development.

Key words: NT2/D1 cells, Wnt signaling, quercetin, LiCl

INTRODUCTION

NT2/D1 is the most widely characterized human EC (embryonal carcinoma) cell line (Andrews, 1984). Due to its similarity to human embryonal stem cells (ES) (Freemantle et al., 2002) and their potential to differentiate into CNS (central nervous system) neurons upon treatment with RA (retinoic acid), the NT2/D1 cell line provides an excellent model system for studying human neural differentiation (Misiuta et al., 2006). It was shown previously that the expression of multiple Wnt signaling molecules is changed during neuronal differentiation of NT2/D1 cells (Katoh, 2002).

Wnt genes function in numerous cellular activities such as cell fate determination, patterning, and migration in embryogenesis, apoptosis, etc (Reya and Clevers, 2005). Mutations in Wnt genes or components of the Wnt pathway lead to specific developmental defects, while various human diseases are caused by abnormal Wnt signaling (Logan and Nusse, 2004; Willert and Jones, 2006). In addition, Wnt signaling is critical for controlling the initiation and progression of many types of cancer (Reya and Clevers, 2005).

Wnt signaling is initiated when Wnt ligands engage their cognate receptor complex consisting of a receptor of the Frizzled family and a member of the LDL receptor family (Reya and Clevers, 2005). The central player in the canonical Wnt signaling pathway is a cytoplasmic protein β-catenin whose stability is regulated by the destruction complex (Cadigan and Liu, 2006; Mareel et al., 1997). In the absence of Wnt signals, newly synthesized β-catenin is trapped in a destruction complex consisting of adenomatous polyposis coli (APC) and axin. Casein kinase 1a (CK1a) and glycogen synthase kinase-3b (GSK-3b) reside in the

destruction complex and phosphorylate β-catenin, targeting it for proteasomal degradation (Reya and Clevers, 2005). When Wnt receptors are engaged, the kinase activity of the destruction complex is inhibited by a mechanism involving Dishevelled (Dsh) protein. This leads to the accumulation of cytoplasmic β-catenin and its translocation into the nucleus where it interacts with TCF/LEF transcription factors and activates Wnt target genes (Eastman and Grosschedl, 1999; Kormish et al.; Park et al., 2005).

In the last couple of decades, substantial efforts have been made towards studying the importance of the Wnt signaling pathway in numerous cellular processes. One of the approaches is to modulate the Wnt pathway by blocking or mimicking *Wnt*/β-catenin-signaling components. For instance, quercetin has been frequently used as an inhibitor of the Wnt pathway (Park et al., 2005; Tomasoni et al.). This flavonoid, commonly present in plants, inhibits β-catenin/TCF signaling by decreasing the nuclear β-catenin and TCF proteins (Park et al., 2005). The mechanism of Wnt pathway inhibition is probably responsible for quercetin's antitumor activity. It has been used in clinical trials in cancer patients (Ferry et al., 1996). On the other hand, previous studies have demonstrated that lithium chloride, a simple cation used for the treatment of bipolar disorder, mimicked the effect of Wnt signaling (Jope, 2003; Manji et al., 2000). This is accomplished by inhibition of GSK-3β, which leads to the accumulation of β-catenin and increased transcription of its target genes (Manji et al., 2000; Misiuta et al., 2006).

The ultimate responses to modulations of Wnt/β-catenin signaling are observed as changes in gene expression. In this regard, numerous target genes downstream of Wnt/β-catenin have been characterized in diverse types of cells and used as indicators of Wnt signaling activity (Huang and He, 2008; Macdonald et al., 2007; White et al., 2007). One of the genes activated by the β-catenin/TCF pathway is *c-myc*, whose expression is widely used for monitoring the up- or downregulation of Wnt signaling (Park et al., 2005)

P53 is a tumor suppressor protein with strong antiproliferative and antiproapoptotic activities. In many cell types, p53 controls apoptosis and regulates cell cycle arrest at G1 and G2 (Lin et al., 1992; Sugimoto et al., 2006; Vousden, 2006). The linkage between p53 and Wnt signaling has been reported previously in mESCs and cancer cell lines (Ilyas, 2005; Lee et al., 2010). Wnt signaling regulates p53 expression by increasing p53 levels in some cell types and decreasing p53 in others (Mao et al., 2001). In addition, it has been shown that the tumor suppressor protein p53 is involved in the regulation of Wnt signaling. p53 activates the expression of E3 ligase of β-catenin and simultaneously regulates the expression of many genes in the Wnt signaling pathway (Tell et al., 2006). In addition, interaction between p53 and GSK3b links the Wnt pathway with the Akt/PKB cell survival pathway. The balance between Wnt signaling, p53 and Akt may mediate whether a stimulated cell undergoes apoptosis, cell cycle arrest or transformation (Tell et al., 2006). Accordingly, the expression of p53 is used for the assessment of apoptosis-mediated cell death.

In this study, we used quercetin and lithium chloride to investigate modulations of the Wnt signaling pathway in the NT2/D1 cell line. First, we optimized conditions for NT2/D1 cell treatments with quercetin and lithium chloride and assessed their cytotoxic effects on NT2/D1 cell viability and proliferation rate. Our results showed that the induction of cell death by quercetin and LiCl is p53-dependent in NT2/D cells. We also examined the degree of Wnt signaling modulations under these experimental conditions by checking the expression of *c-myc*, a well-known Wnt-signaling target gene. These analyses allowed us to determine the optimal concentrations of quercetin and lithium chloride for the modulation of canonical *Wnt* signaling in NT2/D1 cells. We have identified the dosages of quercetin and LiCl that effectively inhibit or stimulate Wnt signaling but have mild effect on cell viability and proliferation.

MATERIALS AND METHODS

Cell culture

NT2/D1 cells were maintained as described (Andrews, 1984). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% Glutamax (Gibco), 1% antibiotic (penicillin-streptomycin; Sigma) and 0.1% antimycotic (amphotericin; Gibco) at 37°C under humidified 10% CO_2 and 91% air at 1 atmosphere.

MTT test

NT2/D1 cells were plated in triplicate in 96-well microtiter plates at a density of $5x10^5$ cells and incubated in the presence of 100 µl of medium supplemented with increasing concentrations of quercetin (40 µM, 70 µM and 100 µM; Sigma) or LiCl (7 mM, 10 mM and 20 mM; Sigma Aldrich). After 24 h, the medium was removed and the cells were incubated in 0.5 mg/ml MTT for 2 h at 37°C. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT to insoluble purple formazan crystals at a rate that is proportional to cell viability (Tada et al., 1986). After removal of the MTT media, 100 µl of DMSO (Sigma) was added to each well, to lyse the cells and solubilize the formazan crystals formed. Absorbance was measured at 620 nm using a plate reader. Untreated negative controls were run together with treated cells.

Western blot analysis

Whole cell lysates were prepared from either NT2/D1 cells alone, or cells treated for 24 h with different counteractions of quercetin or LiCl (as described in the previous section) using NP-40 buffer [20 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 1% NonidetP-40, 10% glycerol and Protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)]. Western blots were performed using anti c-myc (9E10) (Santa Cruz Biotechnology), anti-p53 (DO1) (Gene Spin), anti α-tubulin (DM1A) (Calbiochem), and anti-GAPDH (AM20337PU-S) (Acris Antibodies, Inc).

RESULTS AND DISCUSSION

Quercetin inhibits NT2/D1 cells proliferation in a p53-dependent manner

In order to assess the effect of quercetin on the viability and proliferation of NT2/D1 cells, the cells were treated with three different concentrations of quercetin, 40 µM, 70 µM and 100 µM, for 24 h. As shown in Fig. 1A, 40 µM quercetin had no effect on NT2/D1 cell proliferation, while higher concentrations (70 µM and 100 µM) significantly decreased the percentage of viable cells by approximately 40-60%, in a dose-dependent manner (Fig. 1A). There is no literature data for NT2/D1 cell viability after quercetin treatments but our results are similar to data obtained with other cancer cell lines. Numerous studies have reported that quercetin inhibits cell proliferation in a time- and dose-dependent manner in various human cancer cell lines, esophageal (Cheong et al., 2004), lung (Nguyen et al., 2004), colon (Salucci et al., 2002), breast (Choi et al., 2001), prostate (Yuan, 2005) and leukemia (Kuo et al., 2002). Quercetin shows chemopreventive activity and prevents the development of cancer in a variety of laboratory animal models, such as AOM (azoxymethane)-induced colon cancer in rats and mice, DMBA (dimethylbenz[*a*]anthracene)- and NMU (*N*-nitrosomethylurea)-induced mammary tumors in rats (Deschner et al., 1993; Verma et al., 1988; Yang et al., 2000). In addition, quercetin is used in clinical treatments in cancer patients (Ferry et al., 1996).

To investigate whether the decrease in the proliferation rate of NT2/D1 cells treated with quercetin was result of *apoptosis*-mediated cell death, we assayed the expression of p53, an apoptosis mediator (Fig. 1B). The tumor suppressor gene p53 is regarded as a key factor in a balance between cell survival and cell death by the regulation of both the G1 and G2/M portions of the cell cycle (Agarwal et al, 1995). Our results show that the p53 protein level *significantly increased* in NT2/D1 cells treated with 70 µM and

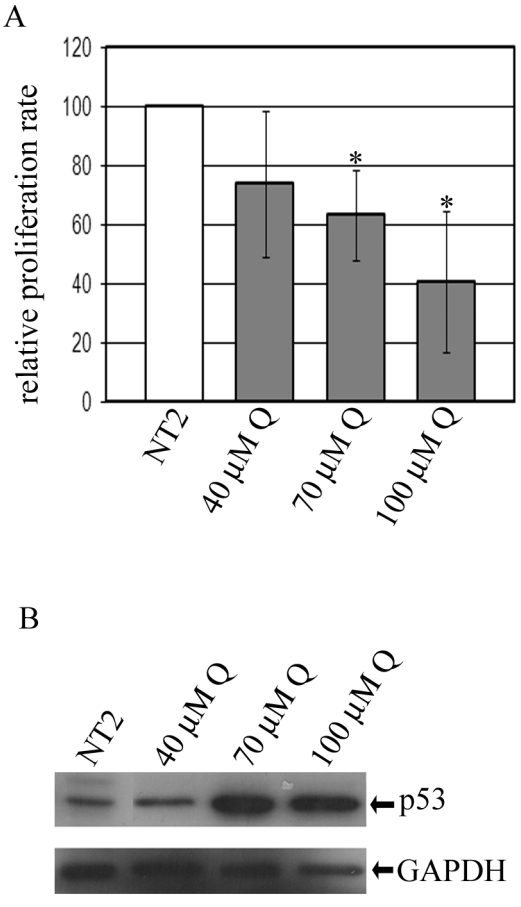

Fig. 1. Quercetin decreases the proliferation rate of NT2/D1 cells in a p53-dependent manner.
(A) MTT proliferation assay of NT2/D1 treated with 40, 70 and 100 μM quercetin for 24 h. The results are shown as percentages of the negative control, untreated NT2/D1 cells. Values are presented as the means ±S.E.M. of at least three independent experiments. Mean values of relative proliferation rates were compared using Student's *t* test. Values of p<0, 05 are presented by *. (B) Western blot analysis of p53 protein expression in NT2/D1 cells treated with 40, 70 and 100 μM quercetin for 24 h. The level of GAPDH was used as a control for equal amounts of input proteins.

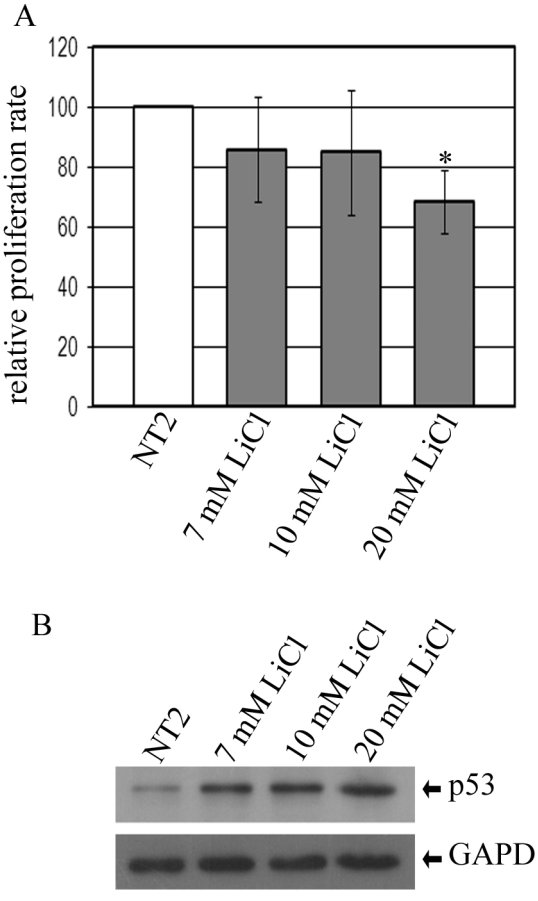

Fig. 2. LiCl decreases the proliferation rate of NT2/D1 cells in a p53-dependent manner.
(A) MTT proliferation assay of NT2/D1 treated with lithium chloride-7, 10 and 20 mM, for 24 h. The results are shown as percentages of the negative control, untreated NT2/D1 cells. Values are presented as the means ±S.E.M. of at least three independent experiments. Mean values of relative proliferation rates were compared using Student's *t* test. Values of p<0, 05 are presented by *. (B) Western blot analysis of p53 protein expression in NT2/D1 cells treated with 7, 10 and 20 mM lithium chloride for 24 h. The level of GAPDH was used as a control for equal amounts of input proteins.

100 μM quercetin (Fig. 1B). These data indicate that quercetin induced p53-mediated cell cycle arrest and apoptosis in NT2/D1cells.

The same effect was observed in human breast cancer MDA-MB-453 cells (Choi et al., 2008) and HepG2 cells (Choi et al., 2008): quercetin significantly inhibited cellular proliferation and induced cell cycle arrest and apoptosis. The molecular mechanism that underlies quercetin-induced cell cycle arrest and apoptosis in HepG2 cells is p53 stabilization at both mRNA and protein levels (Tanigawa et al., 2008).

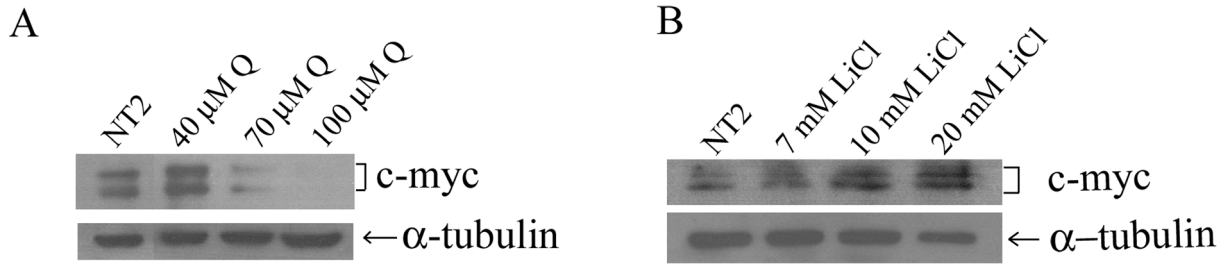

Fig. 3. Western blot analysis of c-myc protein expression in NT2/D1 cells treated with: (A) 40, 70 and 100 μM quercetin and (B) 7, 10 and 20 mM lithium chloride for 24 h. The level of α-tubulin was used as a control for equal amounts of input proteins.

Lithium chloride inhibits NT2/D1 cells proliferation in p53-dependent manner

In the majority of previous studies, NT2/D1 cells were treated with low, clinically applicable doses of LiCl (0,125 mM-1 mM) because of its therapeutic relevance in bipolar disorder treatment. Treatment with low LiCl doses for 24 h significantly increased NT2/D1 cell proliferation, while concentrations around ~10 mM showed no difference in the proliferation rate compared to untreated cells. In our experiments, NT2/D1 cells were treated with three concentrations of LiCl, 7 mM, 10 mM and 20 mM, for 24 h. The obtained data support previously described results (Hill et al., 2008; Misiuta et al., 2006) since the treatment of NT2/D1 cells with 7 mM and 10 mM LiCl was observed not to have an effect on the cell proliferation rate (Fig. 2A). Literature data showed that a concentration of LiCl ranging from 10 mM to 100 mM exhibited high cytotoxity with a significant decrease of NT2/D1 cell proliferation with IC_{50} that is about 70 mM (the concentration of an agent that gives a half-maximal response). In agreement with these data, we have shown that 20 mM LiCl significantly decreased the NT2/D1 cell proliferation rate (approximately 30%) (Fig. 2A). Unlike quercetin, lithium chloride can induce diametrically opposite effects on cell proliferation in different cell types. It has been reported that lithium chloride promotes proliferation and has antiapoptotic, especially neuroprotective, effects in many experimental settings (D'Mello et al., 1994; Hongisto et al., 2003; Kang et al., 2003; Welshons et al., 1995).

However, lithium is not always a neuroprotectant (Song et al., 2004). It promotes apoptosis that is mediated by a death domain-containing receptor and facilitates Fas-induced caspase activation in neural cells (Song et al., 2004). The factors responsible for these variations in cell response are unknown but may be influenced by differences in the underlying genetic background of different cell lines.

In addition, lithium chloride can also induce apoptosis in certain cell types including COS7 monkey kidney cells and a variety of human cells, e.g. HL-60 promyelocytic leukemia cells, 293 embryonic kidney cells, K562 erythroleukemia cells and immature cerebellar granule cells (D'Mello et al., 1994; Madiehe et al., 1995; Tang and He, 2003; van Gijn et al., 2001).

The main cellular target of lithium chloride is GSK-3, a kinase that phosphorylates several cellular substrates, including Mdm2 (Fiol et al., 1994; Kulikov et al., 2005; Saksela et al., 1992), a ubiquitin ligase of the p53 tumor suppressor protein (Brooks and Gu, 2006). Accordingly, inhibition of GSK-3 leads to the accumulation of p53 (Kulikov et al., 2005). Since p53 has strong antiproliferative and proapoptotic activities (Vousden, 2006), we wanted to check whether the lithium-induced decrease in the proliferation rate of NT2/D1 cells is p53-dependent.

Our results suggest that LiCl induces a p53-dependent decrease in the NT2/D1 cell proliferation rate since all three concentrations of LiCl increased the p53 protein level in the treated NT2/D1 cells (Fig. 2B). Literature data show that LiCl induces p53 stabi-

lization by affecting the levels of protein, not mRNA, in primary endothelial cells, leading to G2/M arrest of these cells (Hossein et al., 2012). Also, lithium induced an increase in p53 expression in human melanoma cells (Smalley et al., 2007).

Quercetin and lithium chloride modulate Wnt signaling in NT2/D1 cells

Quercetin has been recognized as potent inhibitor of β-catenin/Tcf signaling. Its inhibitory mechanism is related to the decreased β-catenin and Tcf-4 protein levels in the nucleus, which lead to the downregulation of target genes. In order to investigate whether applied treatments with quercetin modulate the canonical Wnt signaling pathway in the NT2/D1 cell line, we examined the expression of *c-myc*, a well-known β-catenin/Tcf signaling target gene. Our results show that increased concentrations of quercetin downregulate c-myc protein expression in a dose-dependent manner in NT2/D1 cells (Fig. 3A), suggesting that this agent efficiently inhibits the β-catenin/Tcf signaling pathway. At 40 μM quercetin no significant difference in the c-myc protein level was detected compared to untreated cells. Higher quercetin concentrations (70 and 100 μM) decreased and completely abolished c-myc expression (Fig. 3A).

These results are in concordance with previously published data. Quercetin at concentrations ranging from 40 μM to 100 μM significantly decreased c-myc protein expression in a dose-dependent manner in the human gastric carcinoma cell line, MGC-803 (Wang et al., 2006). In SW 480 colon cancer cells, 100 μM quercetin treatment decreases c-myc mRNA and completely abolishes c-myc protein expression (Park et al., 2005).

In our experimental setting, LiCl increased c-myc protein expression in a dose-dependent manner (Fig. 3B). The concentration of 7 mM LiCl has no effect on c-myc expression in NT2/D1 cells, while 24 h treatment with 10 and 20 mM LiCl significantly increased the c-myc protein level (Fig. 3B). It has been previously shown that treatment of Caco-2 and SW480 cells with LiCl resulted in significant increases in c-myc mRNA expression (Brookes et al., 2008). In addition, LiCl acting as a GSK 3b inhibitor prevents the phosphorylation of c-myc and abrogates its proteasomal degradation. This leads to an increase in c-myc protein in HCT116 cells. In three human NSCLC (non-small-cell lung cancer) cell lines, treatment with LiCl increased the expressions of c-myc at both the mRNA and the protein levels (Li et al., 2011).

In this study we demonstrated that quercetin and lithium chloride significantly modulate Wnt signaling in NT2/D1 cells and could be used as potent and efficient modulators of the canonical Wnt signaling pathway in these cells. According to our data, the concentrations of these agents that efficiently modulate Wnt signaling are 70 μM for quercetin and 20 mM for LiCl. At a concentration of 70 μM, quercetin decreased the NT2/D1 cell proliferation rate by approximately 30% and significantly reduced the c-myc protein level. At a quercetin concentration of 100 μM, c-myc protein expression was completely reduced, indicating that Wnt signaling is efficiently inhibited. However, at this concentration quercetin cytotoxicity was very high, with a NT2/D1 cell viability of 40%.

The concentration of 20 mM LiCl considerably increased the c-myc protein level with a 30% decrease in the proliferation rate of NT2/D1 cells. On the other hand, at concentrations of 7 mM and 10 mM, LiCl displayed no effect on the cell proliferation rate (low cytotoxity) while Wnt signaling activation was low sinca small increase in the c-myc protein level was detected.

The canonical Wnt signaling pathway has appeared as a critical regulator of stem cell differentiation. Also, it has been previously shown that the expression profile of multiple Wnt genes is dramatically changed during the early phase of neuronal differentiation of NT2/D1 cells (Katoh, 2002). The sum effect of these changes likely determined the fate of NT2/D1 cells: self-renewal or differentiation. Since the retinoic acid induction of NT2/D1 cells is a good *in vitro* model system for human neural differentia-

tion, studying modulation of Wnt signaling in NT2/D1 could contribute to a better understanding of the mechanisms involved in neural stem cell maintenance and human neural development. Our results show that quercetin and lithium chloride treatments of NT2/D1 cells are efficient methods for Wnt signaling modulations in NT2/D1 cells. Future studies will reveal how these modulations affect the expression of genes involved in retinoic acid-induced neural differentiation of NT2/D1 cells.

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