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Anti-proliferative effect of Kv1.3 blockers in A549 human lung adenocarcinoma *in vitro* and *in vivo*

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ABSTRACT

Voltage-gated potassium (Kv) channels are widely expressed in the plasma membranes of numerous cells and contribute to a variety of cellular functions in both excitable neuronal cells and non-excitable epithelial cells. Recently, it has been demonstrated that Kv channels are associated with the proliferation of several types of cancer cells. In the present study, we investigated the effects of suppression of Kv1.3 expression on cell proliferation and cell cycle progression in human lung adenocarcinoma, A549 cells. Treatment with margatoxin (MgTX), a selective blocker of Kv1.3 or short hairpin RNA (shRNA) against Kv1.3, significantly blocked A549 cells' proliferation. In addition, selective inhibition of Kv1.3 significantly increased expression level of $p21^{Waf1/Cip1}$ and significantly decreased the expression level of Cdk4 and cyclin D3. We also applied the MgTX into a xenograft model using nude mice, and MgTX caused a reduction of tumor volume when it was injected into the tumor tissues. These results suggest that Kv1.3 may serve as a novel therapeutic target for lung adenocarcinoma therapy.

1. Introduction

The function of voltage-gated K^+ (Kv) channels has been well established as the regulator of membrane potential and neuronal activity in excitable cells (Hille, 2001; Gutman et al., 2005). According to recent reports, Kv channels are also expressed in non-excitable cells (Chandy et al., 2004; DeCoursey et al., 1985; Lee et al., 2003) and contribute to various cellular functions such as oxygen sensing, cell proliferation and apoptosis (Kunzelmann, 2005; Lang et al., 2005; O'Grady and Lee, 2005; Pardo, 2004; Wang et al., 2002; Wang, 2004). It had specifically been suggested that Kv channels were over-expressed in cancer cells compared to normal cells (Abdul et al., 2003; Cherubini et al., 2000; Hemmerlein et al., 2006; Jang et al., 2009a, b; Lastraioli et al., 2004; Ousingsawat et al., 2007; Smith et al., 2002) and that they were involved in cell proliferation and control of cell cycle (Annarosa and Andrea, 2006; Hedrich and Becker, 2006). For instance, an absent or low staining of Kv1.3 and Kv4.1 protein was observed in human normal breast tissues while increased expression of Kv1.3 and Kv4.1 appeared in human breast cancer tissues (Abdul et al., 2003; Jang et al., 2009a,b). Several pieces of evidence demonstrate that Kv channels regulate cell cycle progression (Chittajallu et al., 2002; Crociani et al., 2003; Czarnecki et al., 2003; Ghiani et al., 1999; Roura-Ferrer et al., 2008; Villalonga et al., 2008; Wonderlin and Strobl, 1996). Several subtypes of Kv channels, including Kv1.3 (Chittajallu et al., 2002) and Kv1.5 (Villalonga et al., 2008) regulate the expression of cyclin or cyclin-dependent kinase inhibitors (Ghiani et al., 1999). However, the number of studies examining the downstream signaling pathway of Kv channel blockade is limited and signaling pathway is largely unknown.

Margatoxin (MgTX), purified from the toxin of *Centruroides margartatus* and the action of selective blockade against Kv1.3, is well-defined in mammalian cells (Garcia-Calvo et al., 1993; Helms et al., 1997; Knaus et al., 1995; Koo et al., 1997). Although MgTX shares a sequence homology and structural similarity with previously demonstrated K^+ channel blockers, such as charybdotoxin and iberiotoxin, it has a high affinity and specificity against Kv1.3 (Garcia-Calvo et al., 1993; Lin et al., 1993). Moreover, the pharmacokinetic characteristics, such as $t_{1/2}$ of MgTX, were previously identified using *in vivo* study (Koo et al., 1997).

The roles of cyclin, Cdk and their inhibitors on the regulation of cell cycle have been well characterized (Johnson and Walker, 1999; Nigg, 1995). Cyclin D binds to Cdk4 and $p21^{Waf1/Cip1}$ inhibits the cyclin D/Cdk complexes when expressed with a high concentration (Johnson and Walker, 1999). Also, cyclin D3, Cdk4 and $p21^{Waf1/Cip1}$ are critical factors in determining the regulation of the G1 phase progression of cell cycle (Nigg, 1995).

In the present study, we examined the anti-proliferative role of Kv1.3 in human lung adenocarcinoma, A549 cells *in vitro* and *in vivo* using MgTX or short hairpin RNA (shRNA) targeting Kv1.3. Furthermore, we

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examined underlying mechanisms of anti-proliferative effects induced by MgTX or shRNA-Kv1.3.

2. Materials and methods

2.1. Cell culture

A549, human lung adenocarcinoma cell line, was cultured in RPMI 1640 medium Welgene, Daegu, Korea) with 10% fetal bovine serum (Welgene, Korea) and 1% antibiotic–antimycotic solution (Sigma, St. Louis, MO). The condition of culture was kept in humidified 95% air–5% CO_2 at 37 °C, and the medium was changed every other day.

2.2. Total RNA extraction and RT-PCR

Total RNA was extracted using RNAiso Plus (Takara Bio, Otsu, Japan) following the manufacturer's instructions. The purity of extracted RNA was measured by UV spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA) and 2 μg of total RNA was used to synthesize cDNA using a random hexamer and M-MLV reverse transcription kit (Promega, Madison, WI) in a 20 μl volume. To amplify the target mRNA, the PCR reaction was performed with 2 μl of cDNA, gene specific primers (forward: 5′-GTACTTCTTCGACCGCAACC-3′, reverse: 5′-ACCAGCAGTTCGAAGGAGAA-3′) and 1× GoTaq® green master mix (Promega) under the following conditions: initial denaturation at 94 °C for 5 min, cycling (35 cycles) at 94 °C for 40 s, 60 °C for 40 s and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Primers synthesized at Bioneer (Bioneer Corporation, Daejeon, Korea). PCR products were electrophoresised on 1.5% agarose gel and purified using the AccuPrep® Gel Purification Kit (Bioneer). All purified PCR products were confirmed by sequencing (COSMO co, Ltd., Seoul, Korea).

2.3. Transformation and purification of Kv1.3 shRNA plasmid

A SureSilencing™ shRNA plasmids (SABiosciences, MD, USA) were used to inhibit expression of Kv1.3. Four types of shRNA vector, inserted different sequences against Kv1.3 (shRNA-Kv1.3), and negative control shRNA vector that contains a scrambled artificial sequence were transformed into competent cells (iNtRon Biotechnology). The next day, a single colony was picked and cultured in liquid LB medium at 37 °C for 16–20 h. *E coli* cell suspensions were harvested and purified using a transfection-grade plasmid midi kit (Qiagen) following the manufacturers' instructions. Purified plasmid DNAs were confirmed by DNA sequencing and Pst1 restriction enzyme digestion (Enzynomics™, Daejeon, Korea).

2.4. Transient transfection of Kv1.3 shRNA and cell proliferation assay

Approximately, 7×10^4 cells/ml of A549 was prepared in a 96-well plate or 6-well plate containing 10% FBS and 1% antibiotic–antimycotic solution. The next day, cells were transfected with shRNA-Kv1.3 plasmid DNA (0.2 μg of each sequence) using lipofectamine™ 2000 reagent (0.25 μg) (Invitrogen, Carlsbad, CA) or 1 nM MgTX (SantaCruz Biotechnology, Inc. Santa Cruz, CA) was treated. At 4 h post-transfection, the medium was replaced by RPMI 1640 medium supplemented with 10% FBS and 200 μg/ml of G418 antibiotics (Biosesang, Gyeonggi, Korea). Cells were harvested at 24 h post-transfection or MgTX treatment and used for flow cytometry analysis and Western blot analysis. For cell proliferation assay, 0.5 mg/ml methylthiazoltetrazolium solution (Sigma) was added at 24 h post-transfection and incubated at 37 °C for 4 h until formation of MTT formazan. Next, formazan was dissolved using 200 μl of dimethyl sulfoxide (Sigma) and the optical density was measured at 490 nm using an Emax® microplate reader (Molecular Devices, CA, USA).

2.5. Analysis of cell cycle and apoptosis using flow cytometry

After treatment with MgTX or shRNA-Kv1.3, cells were fixed in 70% ethanol, washed with ice-cold PBS and incubated with 50 μg/ml of RNase A solution (Amresco, OH, USA) at 37 °C for 30 min. To detect the DNA contents, 40 μg/ml of propodium iodide (PI) (Sigma) was added. In addition, annexin V and PI double staining were used for the

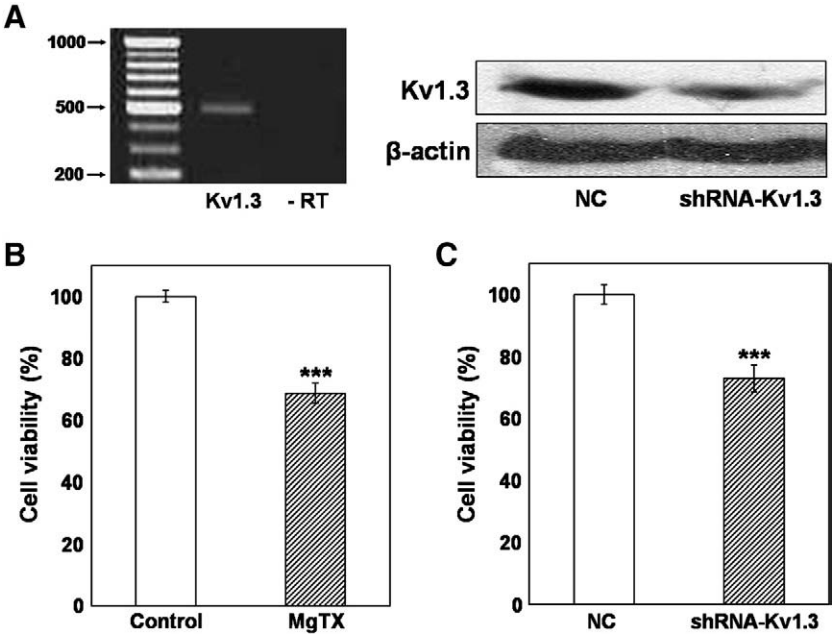

Fig. 1. Effect of Kv1.3 blockade using MgTX and shRNA-Kv1.3 on cell viability of A549 cells. The Kv1.3 mRNA was identified in A549 cells. PCR was performed using total RNA isolated from A549 cells and confirmed on 1.5% agarose gel (left panel of Fig. 1A). The knock-down efficiency of shRNA-Kv1.3 in A549 cells was examined by Western blot analysis (right panel of Fig. 1A). β-actin was used as a loading control. The cell viability of A549 cells was significantly reduced using treatment of 1 nM MgTX ($n=3$) (B) and shRNA-Kv1.3 ($n=3$) (C). Data were normalized as the value obtained for the control or negative control shRNA (NC) and presented as menas ± S.E.M. (***$P<0.0001$).