Expression and Activity of Kv1.3 Potassium Ion Channels in Human Ovarian Cancer Cells

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ABSTRACT Objective: To investigate the expression of Kv1.3 potassium ion channels in SKOV3 ovarian cancer cell line and the role in cell proliferation and cell cycle. Methods: RT-PCR and immunocytochemical methods were used to identify the expression of Kv1.3 potassium ion channels in SKOV3 cells. The effects of Kv1.3 potassium ion channels on SKOV3 cells proliferation and cell cycle were detected by MTT and flow cytometry. Results: 4-Aminopyridine was an effective blocker of Kv1.3 potassium ion channels. Different concentrations of 4-aminopyridine inhibited the proliferation of SKOV3 cell and affected the cell cycle in a dose-dependent manner, which increased the percentage of cells in G0/G1, and reduced the percentage of cells in S phase and G_2/M phase. Conclusions: There was expression of the Kv1.3 potassium ion channels in SKOV3 cells and Kv1.3 play an important role in cell proliferation and cell cycle.

Key words: Kv1.3 potassium channels; Ovarian cancer cells; Cell cycle; Proliferation

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Introduction

Voltage-gated potassium ion channels (Kv) are a large and diverse group of structurally and functionally related proteins. In addition to the four original types of Kv channels, namely Kv1, Kv2, Kv3 and Kv4, recent work has identified several more varieties: Kv5, Kv6, Kv8 and Kv9. Each subgroup may contain several members (e.g., Kv1.1, Kv1.6, Kv2.1, Kv2.2, Kv3.1, Kv3.3, etc.), each having slightly different functional properties likely due to structural differences [1].

Cell proliferation and apoptosis (programmed cell death or cell suicide) are two counterparts that share the responsibility for maintaining normal body function. Abnormally enhanced proliferation often causes loss of control of cell growth leading to tumorigenesis or carcinogenesis or cancer formation. Accumulating evidence indicate a crucial role of plasma membrane K^+ channels in the regulating of tumor cell proliferation[2-5]. K^+ channel antagonists inhibit the proliferation of many types of cells, ranging from quiescent lymphocytes stimulated by mitogens to rapidly cycling tumor cells. There are also numerous reports showing that progression through the cell cycle is dependent on ion translocations across the plasma membrane. Recent studies demonstrated that K+ channel activity also a key determinant factor for cell progression through the G1 phase of mitosis in T lymphocytes [6], oligodendrocyte progenitor cells [7], and breast cancer cells [8].

Kv channels have been previously implicated in the proliferation of normal as well as transformed cells. Regulation of growth by Kv channels also has been observed in a variety of malignant cells, and application of 4-aminopyridine, a Kv channel blocker, resulting in a decreasing in tumor cells [9-14]. Kv1.3 channels are sharker-like Kv channel subtypes. The biophysical and pharmacological properties of Kv1.3 have been reported in primary culture of normal rat prostate epithelial cells [15]. More recently, immunohistochemical studies of human breast tumors showed that Kv1.3 expressed at either moderate or high levels in 88% of the tumors, but could not be detected in normal breast tissue[16]. A similar incidence of Kv1.3 immunolabeling (91% at either moderate of high levels of expression) was detected in human colonic carcinoma specimens, but absent in non-cancerous samples of human colon [17]. Although direct functional evidence for Kv1.3 in breast or colonic tumor cell proliferation was not demonstrated in those studies, the increasing in Kv1.3 protein expression strongly correlated with tumorogenesis.

It was all know that ovarian cancer is the sixth most common cancer and cause of death from cancer in women in the world. Little is known about ion channels in ovarian cancer. This study investigated the expression of Kv1.3 potassium ion channels in SKOV3 ovarian cancer cell line and the role in cell proliferation and cell cycle.

1 Methods

1.1 Materials

Culture flasks and culture plates were purchased from Becton Dickinson, NJ, USA. Propidium iodide (PI), 4-aminopyridine (4-AP) and MTT dye were purchased from Sigma, MO, USA. 4-AP were prepared in different concentrations (1, 5, 10, 15, 20mmol/L) in H_2O and stocked at 4℃. Cell culture media, oligo-dT primers, dATP, dCTP, dGTP, dTTP and M-MLV reverse transcriptase were obtained from Gibco-BRL, USA.

1.2 Cell culture

Ovarian cancer cell line SKOV3 was obtained from Basic Medicine Research Institute, Qilu hospital, Shandong University, PR China. Cells were cultured in 90% RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100U/ml peni-

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cillin, 100μg/ml streptomycin and maintained at 37℃ in a humid atmosphere of 5% CO_2 in air.

1.3 RT-PCR

Total RNA was isolated from the cultured SKOV3 cell line by guanidium thiocyanate-phenol-chloroform extraction procedure. mRNA was transcribed into first strand cDNA by oligo-dT primers and M-MLV reverse transcriptase. The PCR primers (M85217) were designed to amplify the RT-generated Kv1.3 cDNAs. The sense primer of Kv1.3 was 5'GGAATGGTGAGGACCGACTT3' and the antisense primer was 5'CAGCCTTTCTTCTTGATGATGC3'. The expected product length was 382bp. The sense primer of GAPDH was 5'CAGGCGTAGGAATATGGAC3'and the antisense primer was 5'CGTGGCTTTACTTGGAGAA3'. The expected length was 221bp. PCR reaction of 20μl contained 1μl cDNA, 2.5mmol/l $Mgcl_2$, 200μmol/l each dNTP, 0.04u/μl Taq DNA polymerase and 1.0μm primers. Conditions of DNA amplification included an initial denaturation step of 5 min at 95℃, and 35 cycles of 30s at 94℃, 30s at 54℃, and 45s at 72℃, and finally 7 min at 72℃. Half of the PCR samples were electrophoresed in a 2% agarose gel and stained with ethidium bromide (0.5μg/ml). Nucleotide sequence of the amplified products was analysed with ABI310 autosequencer.

1.4 Immunocytochemistry

The cells were seeded on glass coverslips and were washed in PBS 24h after incubating with 5% CO_2 at 37℃. Then they were fixed in 4% paraformaldehyde for 30 min at room temperature. After washed in PBS, the coverslips were blocked with 10% goat serum for 30min to avoid nonspecific binding, and subsequently incubated overnight at 4℃ in a moist chamber with 1:200 primary antibodies (anti-Kv1.3 from Chemicon, Temecula, CA). After washed in PBS, the coverslips were incubated for 30 min at 37℃ with horseradish-peroxidase (HRP)-conjugated, goat anti-rabbit antibody (BioRad, Hercules, CA) at a 1:150 dilution. Freshly prepared 3, 3'-diaminobenzidine (DAB, Sigma Fast Tablets, St, Louis, MO) was used as the substrate for HRP. The positive signals were shown with brown-yellow on plasma membrane. Negative controls were run in parallel, replacing the primary antibody with PBS.

1.5 Proliferation assay

MTT assay was used to analyze the effect of 4-AP on SKOV3 cell proliferation. One hundred microliters of cell suspension (1×10^5 cells/ml) was distributed into each well of flat-bottomed 96-well culture plates, and each plate was incubated with 5% CO_2 at 37℃ for 24h. Then 100μl reagent solutions or media at the desired concentrations was distributed into each well. The well containing media served as positive control. Two hundred microliters of the medium alone without cells and reagent was used as negative control. The culture plate was incubated for 48h. Thereafter, 20μl of the MTT dye (5 mg/ml) was added into each well. Four hour later, 150μl of DMSO was added into each well after discarding meida. The absorbance (A) values of each well at 540 nm were read using an automatic multiwell spetrophotometer (Bio-Rad-Coda, Richmond, CA). The negative control well was used for zeroing absorbance. The percentage of inhibit was calculated using the background-corrected absorbance as follows: inhibit rate = 1-A of experimental well/A of positive control well × 100%. Experiments were performed at least three times with representative data presented.

1.6 Flow cytometry

Cells were cultured in triplicate 25-cm^2 flasks in 0.25ml of 90% RPMI-1640 medium and 10% FBS containing 100U/ml penicillin and 100μg/ml streptomycin. The cells were incubated for 72h with or without K^+ channel inhibitors in an incubator with 5% CO_2 at 37℃. After inhibitors treatment, flow cytometry assays were performed on cell populations. Approximately 106 cells were fixed with 1ml ice-cold 70% methanol for 30 min. After fixing, cells were pelleted by centrifugation to remove the fixatives, then they were washed three times with phosphate-buffered saline (PBS) at 4℃, resuspended in 100μl PBS, treated with 100μl ribonuclease (1 mg/ml, Sigma), and stained with PI at a final concentration of 50 μg/ml. The stained cells were stored at 4℃ in the dark and analyzed within 2 h. The stained samples were measured on a FACScan flow cytometer (Becton- Dickinson, San Jose, CA). The data were stored and analyzed using CellQuest software to assess cell-cycle distribution patterns (G_0/G_1, S, and G_2/M phases).

2 Results

2.1 Identification of mRNA for Kv1.3 studied by RT-PCR

RT-PCR was used to confirm the presence of mRNA for Kv1.3 K^+ channels in SKOV3 cells, and channel subtype-specific primers were used for Kv1.3. Fig 1 showed the results of RT-PCR analysis of Kv 1.3 expression in total RNA extracts from SKOV3 cells. The primers designed for the Kv1.3 amplified the fragment of 382 bp as expected.

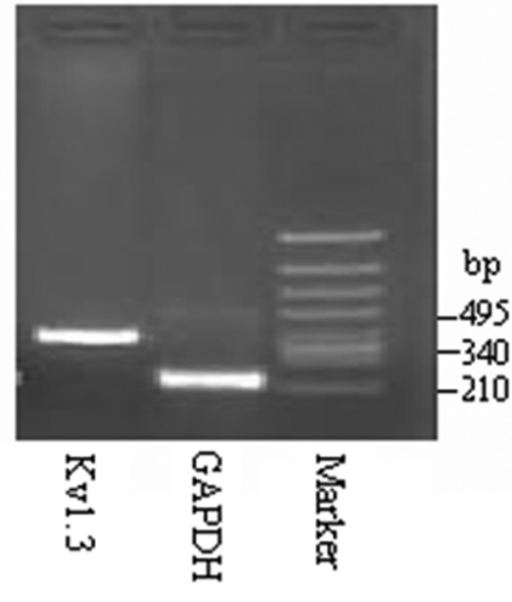

Fig.1 RT-PCR-based detection of mRNA on SKOV3 cells

2.2 Immunocytochemical identification of Kv1.3 channels on SKOV3 cells

The expression of Kv1.3 K^+ channel protein in SKOV3 cells were examined by immunocytochemistry. Fig 2A showed that SKOV3 cell plasma membranes were brown-yellow. Fig 2B showed the negative controls without brown-yellow staining. These suggested Kv1.3 channel protein was expressed on SKOV3 cells.

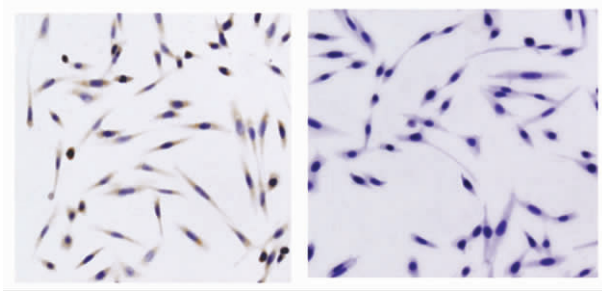

Fig.2 Expression of Kv1.3 on the SKOV3 cell line by immunocytochemistry: A: Cells were incubated with the anti-Kv1.3 antibodies; B: Cells were incubated with PBS as the negative controls

2.3 Effect of 4−AP on proliferation

MTT assay was used to determine the effect of Kv1.3 K^+ channels on cell proliferation. It is known that 4-AP was an effective blocker of Kv1.3 K+ channels. Fig 3 showed that 4-AP can inhibit cell proliferation which was in a dose-dependent manner. Exposing of the SKOV3 cell to 1, 5, 10, 15, 20mmol/L 4-AP, the inhibit rate was 35.0%, 54.6%, 69.1%, 71.2% and 72.8% respectively ($P<0.05$ vs control). These data showed an important role of Kv1.3 channels in the proliferation of SKOV3 cell.

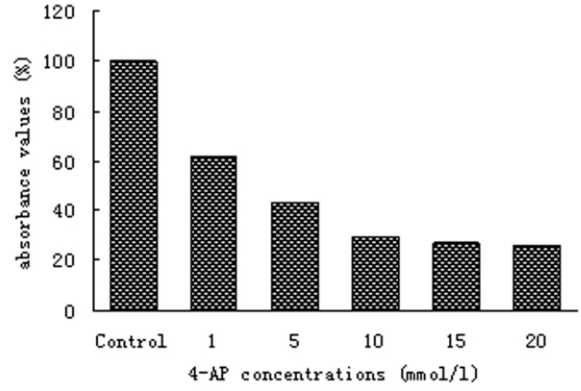

Fig.3 Ability of different concentrations of 4-AP to inhibit cell proliferation. Absorbance values in the absence of 4-AP were normalized to 100%. Each point represents means ± SEM (n = 4-6). $P<0.05$ vs control

2.4 The effect of 4−AP on cell cycle

Flow cytometry was used to examine the effect of 4-AP on cell cycle of SKOV3 cell. The results showed that cell population accumulated in the G_0/G_1 phase and a significantly reduced proportion in the S phase and G2/M phase cells after having been exposed to 4-AP for seventy-two hours (n=6, $P<0.05$ vs control, Table1). These suggested Kv1.3 channels were important for the cell cycle of SKOV3 cell.

Table1 The effect of different concentrations of 4-AP on the progression of cell cycle

Cell cycle (%)	Control	4-AP (mmol /L)				
		1	5	10	15	20
G_0/G_1	39.68	53.28	62.31	78.08	79.11	79.32
S	57.29	45.37	36.25	20.19	20.35	19.56
G_2/M	3.02	1.34	1.43	1.72	0.53	1.11

(n=6, $P<0.05$, each concentration vs. control.)

3 Discussion

Kv channels are a group of plasma membrane ion channels with a key role in controlling repolarization and resting membrane potential in electrically excitable cells. Expression of different types of Kv has been identified in different cells. For example, Kv1.1and Kv1.3 in the MCF-7 breast tumor cell line [12, 16], Kv2 and Kv3 in cervical adrenocarcinoma cell lines and in squamous and esophageal carcinoma cells [18-20]. This study employed RT-PCR and immunocytochemical methods to identify Kv1.3 potassium ion channels in SKOV3 cells. The results showed that there was the expression of the Kv1.3 mRNA in SKOV3 cell and immunocytochemical analysis demonstrated that plasma membrane was labeled by anti-Kv1.3 antibody. These suggested SKOV3 cells express Kv1.3 channels (Fig.1, 2).

Kv channels are involved in the maintenance of vascular smooth muscle tone, glucose-stimulated insulin release by β -pancreatic cells, cell volume regulation, and cell growth. Kv channels were also associated with macrophage functions such as migration, activation, and cytokine production. Moreover Kv channels activity plays an important role in human gastric cancer cells proliferation [10]. Melanoma cells express a diversity of ion-channel types and K^+ channel-blockers have been reported to inhibit melanoma [11] as well as breast cancer growth [12]. Kv channels expressed by rat (Mat-LyLu, AT-2) and human (PC3, LNCaP) prostate cancer cell lines and are involved in cellular growth [13]. Proliferation of human colon carcinoma cell lines SW1116, LoVo, Colo320DM and LS174t was inhibited in the presence of Kv-blockers [14]. The effect

of 4-AP in SKOV3 cell proliferation was investigated. The results showed 4-AP could inhibit cell proliferation and it was in a dose-dependent manner. Exposing of the SKOV3 cell to 1, 5, 10, 15, 20mmol/L 4-AP, the inhibit rate was 35.0%, 54.6%, 69.1%, 71.2% and 72.8% respectively. It is known that 4-AP is an effective blocker of Kv1.3 channels, and 4-AP probably exerted their effect through inhibition of Kv1.3 channels. So these results suggested Kv1.3 channels play an important role in the proliferation of SKOV3 cell. But it is still not understood why K^+ channel activity influences tumor proliferation. Dubois and Rouzaire-Dubois have advanced the hypothesis that K+ channels play a permissive role in cellular proliferation by regulating cell volume and, therefore, the concentration of intracellular solutes, such as Na^+, that are critical for DNA synthesis [21]. Yao X and Kwan HY identified Kv activity in association with cell proliferation and Ca^{2+} influx in colon cancer cells, and then believed activation of K^+ channels hyperpolarizes the membrane, thus increasing the driving force for Ca^{2+} influx and thus interacting with Ca^{2+}-dependent cell cycle control proteins or interfering directly with mitogenic activity, a condition necessary for cell cycle progression [22].

In addition to their effects on cell proliferation, the present data demonstrated that Kv1.3 channels were also important for the cell cycle of SKOV3 cell. Arrest in either G_0/G_1, S or G_2/M regions of the cell cycle was assayed by flow cytometry to assess DNA content and calculate the cell-cycle distribution. This study found that the 4-AP could effect the progression of cell cycle with a 72h treatment of SKOV3 cell. The distribution of G_0/G_1 phase significantly increased, whereas the distribution of S phase and G_2/M phase decreased. During G_1 phase, cells prepare for entry into S phase by performing many essential tasks, such as the uptake of metabolic substrates, the synthesis of RNA and the processing of cell-cycle regulatory signals. The activation of K+ channels might be required for successful completion of one or more of these tasks. Because of the sequential nature of many of the events that occur during G_1 phase, arrest of progression through G_1 phase at a specific site by K^+ channel inhibitor might block multiple downstream events [23]. Kv1.3 channels affected the progression of cell cycle of SKOV3 cell.

In summary, there was expression of Kv1.3 channels in SKOV3 human ovarian cancer cells, and Kv1.3 channels are likely to play an essential role in the physiology of theses cells and, more specifically, in their proliferation and the progression of cell cycle. It was required to design additional experiments to determine if these results can be extended to other ovarian cancer cell types, and to further understand how K^+ channels can interfere with the regulatory pathways involved in cell proliferation and the progression of cell cycle. It should be worthwhile to further explore the possibility of using K^+ channel blockers as a new class of antineoplastic drugs.

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Kv1.3 钾离子通道在卵巢癌细胞株中的表达及活性

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摘要 目的:研究 Kv1.3 钾离子通道在 SKOV3 卵巢癌细胞中的表达及其在细胞增殖和细胞周期中的作用。方法:应用 RT-PCR 和免疫细胞化学鉴别 Kv1.3 钾离子通道在 SKOV3 卵巢癌细胞中的表达。应用 MTT 和流式细胞技术观察 KV1.3 钾离子通道对 SKOV3 卵巢癌细胞增殖及细胞周期的影响。结果:4- 氨基吡啶是 Kv1.3 钾离子通道特异性阻滞剂。不同浓度的 4- 氨基吡啶可以明显抑制 SKOV3 细胞的增殖,并且细胞周期也受到影响。G_0/G_1 细胞比例增加,S 期和 G_2/M 期细胞比例下降。结论:Kv1.3 钾离子通道在 SKOV3 卵巢癌细胞中表达,并且在细胞增殖及细胞周期变换中扮演着重要的角色。

关键词:Kv1.3 钾离子通道;卵巢癌细胞株;细胞周期;增殖

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