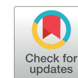
Biochemical and Biophysical Research Communications 504 (2018) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Effects of amiloride on physiological activity of stem cells of human lung cancer and possible mechanism



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ARTICLE INFO

Article history:
Received 11 June 2018
Accepted 25 June 2018
Available online 29 August 2018

Keywords:
Lung cancer
Stem cell
Amiloride

ABSTRACT

Lung cancer is a common malignant tumor, the cancer stem cells (CSCs) were regarded responsible for the development of cancer tissue. The effects of amiloride on lung cancer stem cells and the possible mechanism were not much investigated. In this study, human NCI-H1975 lung CSCs were selected by flow cytometry, and the effects of amiloride at different concentrations (0, 12.5, 25, 50, and 100 μmol/L) were evaluated on proliferation, migration, invasion and apoptosis of CSCs using cell counting kit-8 and Transwell migration assays as well as flow cytometry. Wstern blot analysis was performed to investigate the effect of amiloride on the level of proteins in uPA system, NF-kB pathway, and PI3K-AKT-mTOR pathway in CSCs. As a result, we found that amiloride inhibited proliferation, migration and invasion of lung CSCs, and promoted apoptosis. Further, we found that amiloride decreased levels of target proteins in the uPA system, as well as the NF-kB and PI3K-AKT-mTOR pathways. These results indicated that amiloride could inhibit proliferation, migration and invasion of lung CSCs, and promotes apoptosis, these effects may be related to decreased levels of proteins in the uPA system, the NF-kB pathway, and the PI3K-AKT-mTOR pathway.

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1. Background

Lung cancer is a disease characterized by rapid progression [1]. At present, lung cancer is mainly managed through surgery and supported by radiotherapy or chemotherapy. However, the outcome is usually not satisfactory and mortality of patients with lung cancer remains high [2]. To improve the survival rate of patients suffering from advanced lung cancer, many researchers have devoted their efforts to identify new solutions for lung cancer therapy, develop better validated medicines, and ensure their deployment and uptake in clinical practice. The urokinase-type plasminogen activator (uPA) system mainly consists of uPA, the uPA receptor uPAR (also known as CD87), and the plasminogen activator inhibitors PAI-1 and PAI-2 [3]. The uPA system is involved in growth, invasion, and metastasis of lung cancer cells. The main mechanism involves complex formation between uPA and its receptor uPAR that activates plasminogen into plasmin, and thus, transforms pro-matrix metalloproteinases (pro-MMPs) into activated MMPs, which induce migration and invasion of cancer cells [4]. Further, uPA/uPAR complexes can activate the nuclear factor κB (NF-κB), Ras-mitogen-activated protein kinase (Ras-MAPK), and PI3K-AKT-mTOR signal pathways, thereby promoting tumor development [5–9]. Amiloride, which is a commonly used diuretic in clinical practice, binds to uPA, and previous studies have found that it may play a role in cancer treatment by inhibiting the migration and invasion of tumor cells [10,11]. Moreover, it has been proposed that cancer cells are derived from cancer stem cells (CSCs), possessing self-renewal, multi-lineage differentiation, and high proliferative potential, and it is believed that CSCs are responsible for the development of cancer [12]. Because of this critical role of CSCs in lung cancer, the purpose of our study was to investigate the effect of amiloride on human lung CSCs and determine the possible underlying molecular mechanism.

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https://doi.org/10.1016/j.bbrc.2018.06.138
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2. Materials and methods

2.1. Cell line

The lung cancer stem cell line NCL-H1975 was purchased from the Xiangya Medical College at Central South University.

2.2. Reagents

Amiloride (Sigma, USA), Matrigel (Gibco, USA), and TRIzol (Invitrogen, Carlsbad, CA, USA) were purchased from their respective supplier. RPMI-1640 medium and 10% fetal bovine serum (FBS) were both purchased from Gibco, while Transwell chambers were obtained from Costar Co. (USA), and reverse transcription kit was purchased from Takara Co. (Japan). uPA, uPAR, and MMP9 anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), AKT anti-rabbit antibody was purchased from Cell Signaling Technology (Danvers, MA, USA), and β-actin mouse antibody was obtained from Beijing Zhongjin Jinqiao Co. (Beijing, China).

2.3. Cell selection and culture

The lung cancer stem cell line NCL-H1975 was purchased from the cell bank of Xiangya Medical College at Central South University. Cells were cultured in RPMI-1640 medium containing 10% FBS at 37 °C in 5% CO_2 and 100% relative humidity. Cells in exponential growth phase were selected and cultured. Lung CSCs were sorted using flow cytometry.

2.4. Effect of amiloride on CSC proliferation by CCK-8 assay

Cells in the exponential growth phase were collected as a single cell suspension using 0.25% trypsin, and seeded (1×10^4 cells/well) in a 96-well reaction plate with culture medium containing amiloride at concentrations of 0, 12.5, 25, 50, or 100 μmol/L. All cultures were set up in triplicate. After 12, 24, and 36 h, we added 10 μL CCK-8 and measured absorbance(A) after a 2-h incubation at 37 °C. The inhibitory rate of cell proliferation was determined using Formula (1), followed by calculation of IC50.

$$\text{Inhibitory rate} = [A\ (\text{dosing})\ -A\ (\text{blank})]/[A\ (0\ \text{dosing})\ -A\ (\text{blank})\ *\ 100\% \quad (1)$$

2.5. Effect of amiloride on CSC migration by Transwell migration assay

Transwell chambers were added to wells of a 24-well-plate containing 0.1% BSA-RPMI 1640, followed by the addition of 100 μL cell suspension into the chambers with different concentrations of amiloride (0, 12.5, 25, 50, or 100 μmol/L). The chambers were removed after 12 h, fixed in formalin, and stained using hematoxylin and eosin (H&E). After the cells on the surface of the permeable membrane were removed, samples were processed in neutral balsam. The number of cells migrating from the chambers was counted from five different randomly selected fields of each membrane. Each group was counted for three times.

2.6. Effect of amiloride on invasiveness of CSCs by Transwell migration assay

To evaluate the effect of amiloride on CSC invasiveness, 5 μg Matrigel was added to the surface of a permeable membrane to form a basement membrane, followed by Transwell migration assay as described in section 2.6.

2.7. Detecting apoptosis of stem cells by flow cytometer

Stem cells in the exponential growth phase were collected as a single cell suspension and used to seed wells in a 12-well plate (2×10^5 cells/well). Following a 24-h incubation, amiloride was then added to the wells at different concentrations (0, 12.5, 25, 50, or 100 μmol/L), and cell apoptosis was detected using a flow cytometer 24 h later.

2.8. Effect of amiloride on protein levels of CSCs by western blot

Proteins were extracted using RIPA buffer from stem cells cultured in amiloride at different concentrations for 24 h. Extracted samples were then mixed with 5 × SDS protein loading buffer to a ratio of 1:4 according to their concentration. Following denaturation, samples were transferred to a PVDF membrane by SDS-PAGE, then processed in TBS-T containing 5% skim milk. Next, the membrane was incubated with primary antibody for 2 h at room temperature, followed by another 2-h incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody. For autoradiography, we used ECL followed by X-ray exposure. Images were obtained using an ImageQuant 350 gel protein imaging system. Images were then analyzed using Image J.

2.9. Statistical analysis

Acquired data were analyzed by SPSS Statistics for Windows Version 24.0 (IBM Corp., Armonk, NY, USA) and plotted using GraphPad Prism 6.0. One-way ANOVA test and Student's *t*-test were conducted to compare experimental and control groups. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Isolation of lung cancer stem cells

Lung cancer stem cells were sorted using a flow cytometer, the results are presented in Fig. 1A.

3.2. Effect of amiloride on proliferation of CD133 cells

The CCK-8 assay was performed to evaluate the effect of different concentrations of amiloride (0, 12.5, 25, 50, and 100 μmol/L) on proliferation of lung cancer stem cells. We found that amiloride significantly inhibited proliferation of CSCs in vitro ($P < 0.01$), exhibiting a corresponding step-wise decrease in cell survival rate to increasing concentrations of amiloride (Fig. 1B).

3.3. Effect of amiloride on migration and invasion of CD133 cells

To determine the effect of amiloride on migration and invasion of CSCs, we performed Transwell migration assay, and found that amiloride significantly inhibited migration and invasion of CSCs, and the effect was greater at higher concentrations of the drug (Fig. 1C and D).

3.4. Effect of amiloride on apoptosis of CD133 cells

We used a flow cytometer to detect apoptosis of CSCs treated at different concentrations of amiloride. As are shown in Fig. 2, Our findings indicated that amiloride significantly promoted apoptosis of CSCs, and that the effect was stronger at higher concentrations of the drug.

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