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The inhibitory effect of salinomycin on the proliferation, migration and invasion of human endometrial cancer stem-like cells

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HIGHLIGHTS

- Salinomycin reduced the level of fibronectin expression, which was enhanced in endometrial cancer stem cells.
- Salinomycin induced apoptosis and inhibited Wnt signaling.
- Salinomycin inhibited the proliferation, migration, invasiveness and tumorigenicity of endometrial cancer stem cells.

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ABSTRACT

Goals. We previously demonstrated that side-population (SP) cells in human endometrial cancer cells (Hec1 cells) and in rat endometrial cells expressing oncogenic human K-Ras protein (RK12V cells) have features of cancer stem cells (CSCs). Hec1-SP cells showed enhanced migration and the potential to differentiate into the mesenchymal cell lineage. In this study, we analyzed the association of the epithelial–mesenchymal transition (EMT) with the properties of these endometrial CSCs. We also assessed the effects of salinomycin (a compound with EMT-specific toxicity) on the proliferative capacity, migration and invasiveness of these endometrial CSCs using Hec1-SP cells.

Method. We performed microarray expression analysis to screen for up-regulated genes in CSCs using a set of RK12V-SP cells and -non-SP(NSP) cells and used the Metacore package to identify the Gene GO pathway MAPs involved in the up-regulated genes. To analyze their association with EMT, the expression of several EMT associated genes in Hec1-SP cells was investigated by real time PCR and compared with that in Hec1-NSP cells. We assessed the expression of *BAX*, *BCL2*, *LEF1*, *cyclinD* and *fibronectin* by real time PCR. We also evaluated the viabilities, migration and invasive activities, and tumorigenicities of these SP cells and NSP cells in the presence or absence of salinomycin.

Results. We demonstrated that i) EMT processes were observed in both RK12V-SP cells and Hec1-SP cells, ii) the level of *fibronectin* was enhanced in Hec1-SP cells and salinomycin reduced the level of *fibronectin* expression, iii) salinomycin induced apoptosis and inhibited Wnt signaling, and iv) salinomycin inhibited the proliferation, migration, invasiveness and tumorigenicity of these SP cells.

Conclusion. This is the first report of an inhibitory effect of salinomycin on the properties of endometrial CSCs.

Introduction

Stem cells have a marked self-renewal capacity and are pluripotent. They can be identified in embryonic tissues as well as in normal adult tissues. The existence of cancer stem-like cells (CSCs) has been proposed and CSCs have been identified in leukemia and several solid tumors [1–3]. The properties of CSCs are as follows: i) they possess self-renewal capacity, ii) they can produce progeny cells, iii) they constitute a small minority of neoplastic cells within a tumor, and iv) they possess the developmental potential for expression of multiple specific markers [4]. CSCs are resistant to current cancer treatment, resulting in an increased risk of recurrence.

Side-population (SP) cells are enriched in stem cells and have been isolated and characterized, using fluorescence-activated cell sorting (FACS). The methodology is based on the cells' ability to reduce the intracellular concentration of the fluorescent dye Hoechst 33342 [5]. The identification of SP cells is associated with a high

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expression level of the ATP-binding cassette transporter protein ABCG2/BCRP1. The ATP-binding-cassette (ABC) transporters represent the largest family of transmembrane proteins capable of exporting a wide variety of molecules and structurally unrelated chemotherapeutic drugs from the cytosol. These proteins confer multidrug resistance to CSCs [6,7].

We isolated and characterized SP cells present in human endometrial cancer cells (Hec1 cells) and in rat endometrial cells expressing oncogenic human K-Ras protein (RK12V cells). The SP cells showed reduced expression levels of differentiation markers, long-term proliferative capacity of the cell cultures, self-renewal capacity, enhanced tumorigenicity, and enhanced migration. These findings demonstrate that SP cells have features of CSCs, including the potential to differentiate into the mesenchymal cell lineage [8].

The epithelial–mesenchymal transition (EMT) occurs during normal early embryonic development. This program allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity and invasiveness [9]. EMT is also a key developmental program that is often activated during cancer invasion and metastasis. The EMT program enables cancer cells to disseminate from a primary tumor by losing epithelial characteristics and acquiring a mesenchymal phenotype. Mani et al. have reported a direct link between EMT and the gain of epithelial stem cell properties [10]. Human breast cancer stem cells express Snail and Twist, EMT-inducing transcription factors. When their expression is elevated, the size of the stem cell population is increased and expression levels of mesenchymal markers fibronectin and vimentin are enhanced [10]. Thus, EMT promotes the generation of cancer stem cells.

Salinomycin is a selective inhibitor of CSCs [11]. Salinomycin is an antibacterial and coccidiostatic therapeutic drug. It has been shown to kill breast cancer stem cells in mice at least 100-times more effectively than the commonly used anti-cancer drug paclitaxel. Although several studies demonstrate that salinomycin has an inhibitory effect on proliferation of CSCs, the effects of salinomycin on the properties of endometrial CSCs are unclear.

In this study, we used Hec1-SP cells and RK12V-SP cells to analyze the association of EMT with the properties of endometrial CSCs and the effects of salinomycin on the proliferative capacity, migration and invasiveness of endometrial CSCs.

Materials and methods

Cell lines and cell culture

An endometrial cancer cell line (Hec-1) and a rat endometrial cell line (RENT4) were used in the present study. The Hec-1 cell line was established by Kuramoto et al. from explants of adenocarcinoma of human endometrium [12] and it was a gift from Dr. Kuramoto. RENT4 cells were established by Wiehle et al. [13] and obtained from the European Collection of Cell Cultures (ECACC). Both cell lines were authenticated by Takara Bio Inc. using the short tandem repeat (STR) DNA profiling. The STR profiles of Hec-1 cells were matched to their original profiles. Interspecies contamination was ruled out by the STR profiles in both cell lines. Both cell lines were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum (FBS) + 100 U/mL penicillin, and 100 μg/mL streptomycin.

Isolation of SP cells

To identify and isolate Hec1 SP cells, the cells were dislodged from the culture dishes with trypsin and EDTA, washed, and suspended at a concentration of 1×10^6 cells/mL in DMEM containing 10% FBS. The cells were then labeled in the same medium at 37 °C for 90 min with 2.5 μg/mL Hoechst 33342 dye (Molecular Probes, Eugene, OR), either alone or in combination with 50 μmol/L verapamil (Sigma-Aldrich). Finally, the cells were counterstained with 1 μg/mL propidium iodide (PI) to label dead cells. The cells were then analyzed in a FACS Vantage fluorescence-activated cell sorter (BD Biosciences, San Jose, CA) using dual wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350 nm UV light. PI-positive dead cells were excluded from the analysis.

The SP cells were separated by FACS from the non-SP (NSP) cells and both fractions were seeded in a mesenchymal stem cell maintenance medium (MF medium; Toyobo, Osaka, Japan) containing 10% FBS on collagen-coated 24-well plates (2 cm^2) (Iwaki, Funabashi, Japan). The cells were cultured for two to four weeks. The cells were then transferred to collagen-coated plates (60 mm).

RNA isolation

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Real time quantitative PCR analysis

Real time PCR was carried out using a 7300 Real Time PCR System with SDS RQ Study software (Applied Biosystems). cDNA templates were combined with SYBER Green premix with Rox (Takara Bio) to perform quantitative-PCR reactions. Primers used in this study are shown in Supplementary Table 1. Reactions were carried out for 1 cycle at 94 °C for 5 min; 40 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s; 1 cycle at 94 °C for 1 min; and 1 cycle at 55 °C for 30 s. The transcript level of each specific gene was normalized to GAPDH amplification.

In vitro scratch assay

The SP and NSP cells were cultured on 24-well plates in complete medium. Upon reaching confluence, the medium was replaced with conditioned medium DMEM containing 1% FBS in the presence or absence of salinomycin (1 μM) for an additional 24 h, and the cell layer was wound with 200 μL tips. After 24 h of incubation, the cells were photographed with a BZ-8100 microscope (Keyence, Japan).

Invasion assays

Cell invasiveness was assessed with a BioCoat Matrigel Invasion Chamber kit according to the protocol of the manufacturer (Becton Dickinson Labware, Bedford, MA). We seeded 5×10^4 Hec1-SP or -NSP cells treated with or without salinomycin (1 μM) into the Transwell insert chamber with a filter coated with Matrigel and placed the inserts in the lower chambers filled with 750 μL of DMEM containing 10% FBS. Chambers were incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. Thereafter, we removed the inserts and scraped off the non-invading cancer cells remaining on the upper side of the filter. The cells that had invaded the lower side of the filter were viewed under a Nikon phase-contrast microscope and counted in >10 fields of view at ×200 magnification. The number of cells on the lower side of the filter was normalized to cells using the control chamber without matrigel. The assay was done in triplicate.

In vivo tumor formation assay

We inoculated 1×10^5 cells in Matrigel (BD Matrigel Basement Membrane Matrix High Concentration; BD Biosciences, Bedford, MA) into the subcutaneous connective tissue of five-week-old nude mice (Balb nu/nu). Four to six months after injection of Hec1-SP cells, mice were sacrificed and the tumors excised. Eight mice were injected subcutaneously with 0.26 μM salinomycin and another eight mice were injected with 100 1/4 L DMSO twice a week. The size of the tumor Q3

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