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A possible usage of a CDK4 inhibitor for breast cancer stem cell-targeted therapy

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ABSTRACT

Cancer stem cells (CSCs) are one of the main reasons behind cancer recurrence due to their resistance to conventional anti-cancer therapies. Thus, many efforts are being devoted to developing CSC-targeted therapies to overcome the resistance of CSCs to conventional anti-cancer therapies and decrease cancer recurrence. Differentiation therapy is one potential approach to achieve CSC-targeted therapies. This method involves inducing immature cancer cells with stem cell characteristics into more mature or differentiated cancer cells. In this study, we found that a CDK4 inhibitor sensitized MDA-MB-231 cells but not MCF7 cells to irradiation. This difference appeared to be associated with the relative percentage of CSC-population between the two breast cancer cells. The CDK4 inhibitor induced differentiation and reduced the cancer stem cell activity of MDA-MB-231 cells, which are shown by multiple marker or phenotypes of CSCs. Thus, these results suggest that radiosensitization effects may be caused by reducing the CSC-population of MDA-MB-231 through the use of the CDK4 inhibitor. Thus, further investigations into the possible application of the CDK4 inhibitor for CSC-targeted therapy should be performed to enhance the efficacy of radiotherapy for breast cancer.

1. Introduction

Cancer stem cells (CSCs) or cancer initiating cells/stem-like cancer initiating cells (CICs) were first indentified in human leukemias [1,2]. Since then, CSCs have been identified and characterized in several solid tumors, including colon, prostate, brain and breast cancer [3–8]. CSCs have been shown to be capable of self-renewal and differentiating into other phenotypes of the parent tumor and they are also more tumorigenic when compared to other subsets. The most important feature of CSCs, in terms of cancer therapy, is their resistance to anti-cancer therapy including chemo- and radiotherapy [9–11]. Several possible mechanisms have been proposed to explain the resistance of CSCs to anti-cancer therapies and several CSC-targeted therapies are currently under investigation [12–17]. Differentiation therapy may be one approach to achieve CSC-targeted therapies. Given the characteristics of CSCs, differentiation of CSCs into more mature tumor subpopulations may increase their susceptibility to conventional cancer therapy.

In breast cancer, the $CD24^{-}/CD44^{+/low}ESA^{+}$ population was originally shown to bear CSCs with an enhanced ability to form tumors in immune-compromised mice and they were found to be capable of differentiating into distinct cellular subtypes [8,18,19]. In breast cancer cell lines, the $CD24^{-}/CD44^{+}$ population was characterized as a subpopulation with CSC-like phenotypes [20,21]. Even though $CD24^{-}/CD44^{+}$ is the most widely used marker to characterize breast CSCs, other markers have also been used, including presence of a side population (SP, Hoechst 33342 exclusion), aldehyde dehydrogenase (ALDH) activity and CD133 and $CXCR4^{+}$ [22].

Cell-cycle regulators have been extensively studied as targets for cancer therapy since cell cycle machineries of cancer cells are often deregulated. CDK4, a cyclin-dependent kinase, coordinates the cell-cycle progression by phosphorylating and inactivating Rb protein [23–25]. Given the frequent overexpression of Cyclin D1 in breast cancers, CDK4 may be a possible therapeutic target for breast cancers [26–28].

In this study, we investigated the effect of inhibiting CDK4 activity in relation to preventing breast cancer. In these experiments, we found that a CDK4 inhibitor sensitized MDA-MB-231 cells but not MCF7 cells to irradiation. The difference between the two breast cancer cell lines appeared to be associated with the relative percentage of the CSC-population. Treatment of

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MDA-MB-231 cells with the CDK4 inhibitor differentiated the $CD24^-/CD44^+$ population into a $CD24^+/CD44^+$ population, reduced their mammosphere forming ability and ALDH activity. These results imply that the cancer stem cells were differentiated by the treatment. In contrast, the CDK4 inhibitor did not produce a similar effect on MCF7 cells, which have a $CD24^-/CD44^+$ CSC-population of less than 1%. These results suggest that inhibition of CDK4 activity may be a possible method for achieving cancer stem cell-targeted therapy to enhance the efficacy of radiotherapy.

2. Materials and methods

2.1. Cell culture, cell proliferation assay and mammosphere formation assay

MCF7 and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. A CDK4 inhibitor was purchased from Millipore (Millipore, Billerica, MA, Cat. # 219476). Cell proliferation was measured using a CellTiter96 Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega, Madison, WI, Cat. # G4000) according to the manufacturer's instructions.

In the mammosphere formation assay, MDA-MB-231 cells were incubated with or without 2.5 μM of the CDK4 inhibitor for 24 h, washed and counted. Cells were plated on ultralow attachment plates (Corning Costar Corp., Cambridge, MA) at a density of 4000 viable cells/ml using a serum-free MammoCult Human Medium Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. 7–10 days later, images were obtained.

2.2. Irradiation

Gamma irradiation was performed using a BIOBEAM 8000 (Gamma Service Medical GmbH, Leipzig, Germany). Gamma irradiation was delivered at room temperature using a 77.33TBq ^{137}Cs source at a dose rate of 2.52 Gy/min.

2.3. Clonogenic assay

MCF7 and MDA-MB-231 cells were incubated with or without 2.5 μM of the CDK4 inhibitor for 24 h, washed out and then irradiated. 24 h after irradiation, the cells were harvested, seeded and incubated in 1% agar in DMEM media (containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 12–14 days to allow colony formation. Colonies were stained with crystal violet and counted. The surviving fractions were normalized by setting the percentage of surviving colonies in the control wells to 1.

2.4. Flow cytometry analysis

To measure the Sub-G1 fraction, cells were harvested at 24 h following CDK4 inhibitor treatment, fixed in 70% ethanol, and stained with 40 μg/ml propidium iodide (PI) in the presence of 50 μg/ml RNase A. The DNA content of each sample of 10,000 cells was analyzed on a Becton Dickinson FACS Aria (Franklin Lakes, NJ, USA) using FACSDiva software. The CD24-FITC and CD44-APC antibody (BD Pharmingen, San Diego, CA, USA) were used to measure the $CD24^+CD44^+$ CSC-population. ALDH activity was measured using an ALDEFLUOR Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. Results were analyzed on a Becton Dickinson FACS Aria (Franklin Lakes, NJ, USA) using FACSDiva software.

3. Results

3.1. A CDK4 inhibitor induced similar levels of cell cycle arrest and death on MCF7 and MDA-MB-231 cells

Deregulation of cell-cycle progression is one of the main characteristics of cancer and has been investigated as a therapeutic target for cancer. Thus, many efforts have been devoted to developing compounds that inhibit cell-cycle regulators, such as CDKs. However, many potential inhibitors of cell-cycle regulators may be ineffective due to the presence of inhibitor-resistant cancer cell populations. Several approaches have been developed to overcome inhibitor-resistant cancer cell populations and one potential approach is combination therapy using two different modalities of anti-cancer therapeutics. Thus, in this study, we combined a cell-cycle inhibitor, specifically a CDK4 inhibitor, with irradiation to assess the effects of such a treatment against breast cancer cells.

Two different breast cancer cell lines, MCF7 and MDA-MB-231 cells were used. Both breast cancer cells were treated with the indicated concentration of a CDK4 inhibitor for 24 h and then subject to FACS analysis and a cell proliferation assay (MTT assay). The CDK4 inhibitor treatment inhibited cell proliferation and induced mild cell death in both breast cancer cells (Fig. 1A and B). The CDK4 inhibitor appeared to be more effective against MCF7 cells than MDA-MB-231 cells in terms of inhibiting cell proliferation and induction of cell death.

3.2. Combination of the CDK4 inhibitor with irradiation showed differential effect on MCF7 and MDA-MB-231 cells

In the next step, we combined the CDK4 inhibitor treatment with irradiation. 2.5 μM of the CDK4 inhibitor was used since cell proliferation was found to be reduced by about 50% relative to the control cells for both MCF7 and MDA-MB-231 cells (Fig. 1B). MCF7 and MDA-MB-231 cells were treated with the CDK4 inhibitor for 24 h, irradiated with several doses and then subjected to the clonogenic assay. For MCF7 cells, irradiation alone was found to be more effective than the combination of the CDK4 inhibitor and irradiation (Fig. 2). In contrast, the combination therapy was more effective than irradiation alone for the MDA-MB-231 cells. These results suggest that pre-treatment with the CDK4 inhibitor radiosensitized MDA-MB-231 but not MCF7 cells.

3.3. The CDK4 inhibitor reduced CSC-population of MDA-MB-231 cells

We also attempted to determine why the combination therapy had different effects on MCF7 and MDA-MB-231 cells. Both MCF7 and MDA-MB-231 cells are breast cancer cells but they have different characteristics, where one important difference is the $CD24^-CD44^+$ population, which is known to bear CSCs [20,21]. While investigating the effects of the CDK4 inhibitor, we found that the CDK4 inhibitor differentiated the $CD24^-CD44^+$ population into $CD24^+CD44^+$ population in MDA-MB-231 cells (Fig. 3A). This suggests that the CDK4 inhibitor may differentiate CSC-population of MDA-MB-231 cells. Thus, we further investigated this possibility using more CSC-markers or phenotypes. The effect of the CDK4 inhibitor on the mammosphere forming capacity was examined. MDA-Mb-231 cells were treated with the CDK4 inhibitor for 24 h, washed and then plated in serum-free, low adherence culture. The CDK4 inhibitor reduced mammosphere formation when compared to the control group (Fig. 3B). Aldehyde dehydrogenase activity (ALDH) has also been used as a marker of CSCs, especially

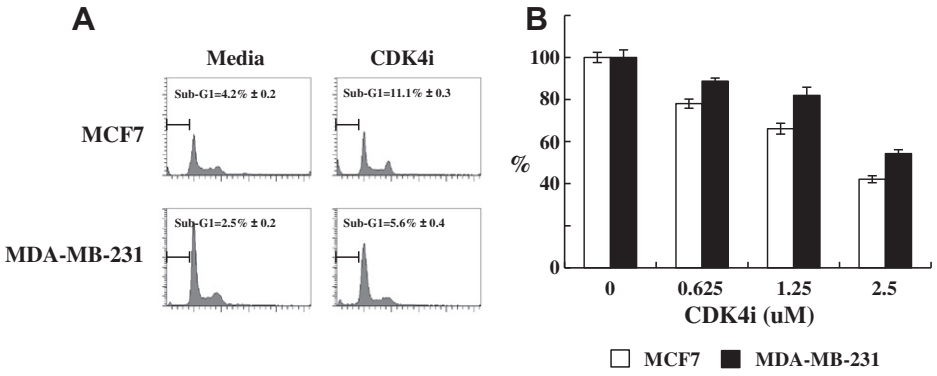

Fig. 1. The effect of the CDK4 inhibitor on cell cycle, cell death and proliferation of MCF7 and MDA-MB-231 cells. (A) MCF7 and MDA-MB-231 cells were treated with 2.5 μM of the CDK4 inhibitor for 24 h. Cells were harvested, fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry to determine cell cycle progression and the sub-G1 population. (B) MCF7 and MDA-MB-231 cells were treated with the indicated concentration of the CDK4 inhibitor for 24 h. Relative cell viability (proliferation) was assessed by using the MTT assay. Data (O.D. 570) were normalized by setting the O.D. 570 of the 0 μM sample of the each group to 100%. Error bars represent the standard deviation.

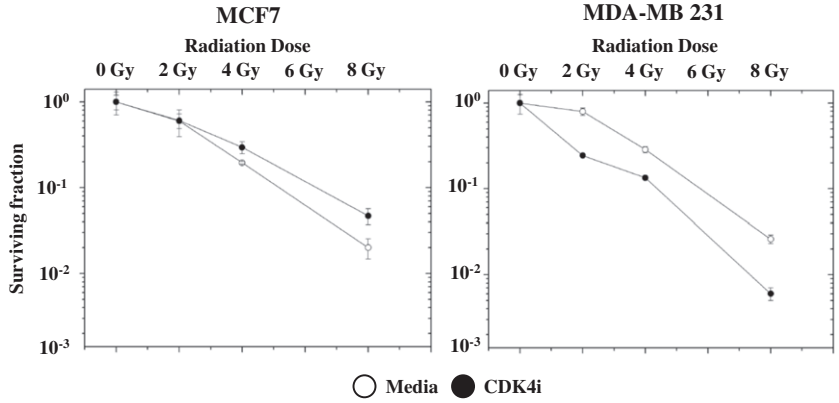

Fig. 2. The CDK4 inhibitor radiosensitized MDA-MB-231 but not MCF7 cells. MCF7 and MDA-MB-231 cells were incubated with or without 2.5 μM of the CDK4 inhibitor for 24 h, washed and then irradiated. 24 h after irradiation, the clonogenic assay was performed. The surviving fractions were normalized by setting the percentage of the surviving colonies in the control wells of each group to 1. Error bars represent standard deviation.

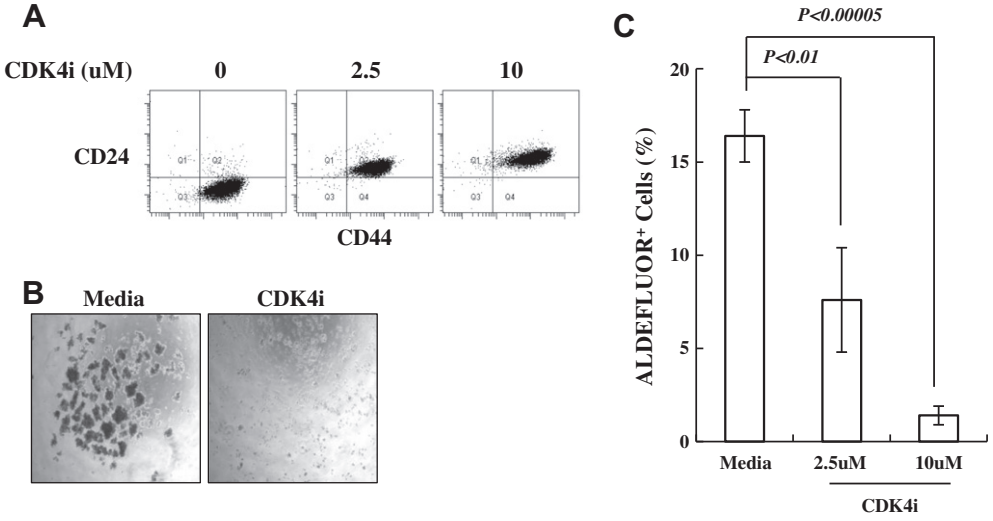

Fig. 3. The CDK4 inhibitor reduced cancer stem cell characteristics of MDA-MB-231. (A) MDA-MB-231 cells were treated with the indicated concentration of the CDK4 inhibitor for 24 h. Cells were stained with CD24-FITC and CD44-APC antibody and then analyzed using a FACS system. (B) MDA-MB-231 cells were incubated with or without 2.5 μM of the CDK4 inhibitor for 24 h, washed and counted. Cells were plated on ultralow attachment plates to allow for the formation of mammospheres using the serum-free MammoCult Human Medium Kit. 7–10 days later, images were obtained. (C) MDA-MB-231 cells were incubated with the indicated concentration of the CDK4 inhibitor for 24 h, ALDH activity was measured using the ALDEFLUOR Kit. The samples were then analyzed on a FACS system. Error bars represent the standard deviation.

for breast cancer cells, and its inhibition has been shown to reduce chemotherapy and the radiation resistance of breast cancer cells [29,30]. We compared the ALDH activity between the control and the CDK4-treated MDA-MB-231 cells using the ALDEFLUOR assay. The CDK4 inhibitor reduced the ALDH activity of MDA-MB-231 cells in a dose-dependent manner (Fig. 3C). Taken together, these results suggest that the CDK4 inhibitor may reduce the CSC-population or the activity of MDA-MB-231 cells.

4. Discussion

Despite the advances in anti-cancer therapeutics, cancers often relapse in a large portion of patients after the initial regression of cancer [14,31]. There may be several possible mechanisms for the cancer recurrence. One of them may be the existence of CSCs which are known to be more resistant to chemo- and radiation-therapy. Thus, it is believed that CSC-targeted therapeutics may improve the efficacy of anti-cancer therapies. Currently, several CSC-targeted strategies are under development [13,14,32]. One potential approach is differentiation therapy, which can be used to increase the susceptibility of cancer cells to anti-cancer therapies by inducing CSCs into mature or differentiated cancer cells. This may be achieved by altering the signaling pathway or modifying the expression of genes involved in differentiation processes.

While studying the effects of a CDK4 inhibitor, we unexpectedly observed that the CDK4 inhibitor radiosensitized MDA-MB-231 but not MCF7 cells and this phenomenon may be associated with the differentiation of the $CD24^{-}CD44^{+}$ CSC-population and reduction of ALDH and mammosphere forming activity of MDA-MB-231 cells. Since CSCs are known to be more resistant to irradiation than the other population, a reduction in the CSC-population by the CDK4 inhibitor may sensitize MDA-MB-231 cells to irradiation. The opposite response was observed in MCF7 after the treatment with the CDK4 inhibitor. MCF7 and MDA-MB-231 cells are breast cancer cell lines but have several different phenotypes and characteristics, such as the percentage of the $CD44^{+}/CD24^{-}$ CSCs population. The $CD44^{+}/CD24^{-}$ CSCs population in MCF7 cells is less than 1%, whereas the $CD44^{+}/CD24^{-}$ CSCs population is more than 80–90% in MDA-MB-231 cells [20]. Thus, the differentiation effect caused by the CDK4 inhibitor may not occur in MCF7. Instead, cell cycle arrest of MCF7 cells by the CDK4 inhibitor at the G1 phase where cells are more resistant to irradiation than at the other cell cycles may decrease the radiosensitivity.

Several markers or phenotypes are currently used to identify CSC-populations. However, the CSC-populations identified by different markers do not always overlap [33]. Thus multiple markers or phenotypes may need to be used to identify CSC-populations. In this study, we used three different methods to evaluate the effect of the CDK4 inhibitor on CSCs. 2.5 μM of the CDK4 inhibitor differentiated most of $CD24^{-}/CD44^{+}$ population to $CD24^{+}/CD44^{+}$ population and inhibited the mammosphere forming activity of MDA-MB-231 cells. However, 2.5 μM of the CDK4 inhibitor decreased the ALDH activity down to ~50% of the control. This difference may be due to the fact that the CSC-populations that were determined using the different markers did not completely overlap. Taken together, we have shown that the CDK4 inhibitor may be used for breast CSC-targeted therapy and furthermore when this treatment was combined with irradiation, an enhanced anti-cancer activity was observed.

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