RADIOSENSITIZATION OF HELA CELLS BY PHOSPHATIDYLINOSITOL-3-KINASE INHIBITORS

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ABSTRACT

Phosphatidylinositol 3-kinases (PI3Ks) regulate a lot of essential cellular processes including cell growth, proliferation, differentiation and survival. It has been found that components of PI3K signalling pathway are frequently altered in human cancers. Moreover, disrupted regulation in PI3K pathway has been established in one-third of human tumors. Upregulated PI3K signalling promotes tumorogenesis and influences conventional therapies. Thus PI3K pathway has emerged as an attractive molecular target for novel anticancer therapies.

Cancer treatment by gamma-radiation aims to kill the cells primarily by the induction of apoptosis due to accumulated DNA damage. Cells exposed to the DNA damaging agents induce cell cycle arrest to allow time for DNA repair and in this way to avoid apoptosis. The ability of tumor cells to avoid apoptosis leads to the development of resistance to the therapy. Therefore, introduction of new therapeutic methods, additionally promote apoptosis, could lead to improved effectiveness of the traditional radiotherapy.

In the present study we examined the mechanism by which the prototype PI3K inhibitors caffeine and wortmannin sensitize HeLa cells to gamma-irradiation. Our results showed that both inhibitors partially abrogated gamma-radiation induced G2/M phase arrest allowing damaged cells to undergo mitosis with unrepaired DNA, driving them to apoptosis and in this way increasing the effect of gamma-radiation on cervical carcinoma cells.

***Key words**: PI3K inhibitors, caffeine, wortmannin, cell cycle arrest*

Introduction

Cervical cancer is the second most common cancer among women according to the most recent estimates of the global cancer incidence, and it remains a significant health care problem throughout the world. Radiation therapy and chemotherapy play a pivotal role in the treatment of advanced stage cervical cancer [1, 2]. Unfortunately, they exhibit high degree of toxicity, low selectivity and have a lot of adverse side effects. Therefore, the finding of agents, capable of enhancing the effect of the chemo- or radiotherapy would permit the application of lower therapeutic doses and hence reduction of the side effects of the therapy.

Ionizing radiation generates a variety of lesions – abasic deoxyribose sites, single and double stranded DNA breaks, DNA-protein crosslinks etc. The treated cells induce cell cycle arrest necessary for DNA repair mechanisms [3]. Typically, cancer cells exposed to gamma-radiation induce cell cycle arrest in late G2 phase of the cell cycle, especially if they have mutant p53 gene or do not express p53 as HeLa cells. Depending on the extent of radiation-induced damage, cells either repair the damage during cell cycle arrest or die. However, gamma-radiation could be inefficient inducer of apoptosis in some type of cells, like cervical cancer HeLa cells, which are considered to be relatively radioresistant [4].

The PI3K/Akt pathway is one of the most powerful intracellular pro-survival signaling systems. It is known that overexpression of PI3K coexists with high-risk oncogenic HPV types in cervical cancer and leads to faster progression of HPV-transformed epithelial cells into invasive tumors. PI-3 kinases are also involved in cell growth, proliferation, transformation and suppression of apoptosis. In recent years, several components form the PI3K/Akt/mTOR signalling pathway

have been used as targets for anticancer therapy. PI3K inhibitors have been broadly studied as potential therapeutic agents against different tumors and some anti-PI3K drugs are currently under investigation in clinical trials [5].

In this study, we set out to analyze the effect of two PI3K inhibitors – caffeine and wortmannin on the in vitro grown cervical cancer cell line HeLa, in combination with the well characterized DNA damaging agent gamma-radiation. Caffeine (methylxantine) is a natural purine alcaloide, component of many popular drinks, most notably tea and coffee. Recently, it has attracted great interest because of its remarkable, dose-dependent multifunctional inhibitory effects on carcinogenesis in cultured cells or animal models. Caffeine has been shown to affect cell cycle control mechanisms, enhance the toxicity of anticancer agents and induce programmed cell death in tumor cells. Moreover, caffeine can inhibit various components of the PI-3 kinase signaling pathway [6]. Wortmannin, which is a natural steroid, also inhibits covalently different members of PI3K family. It represents the first generation inhibitors with highly potent PI3K-inhibitory property [7]. As a consequence of down-regulation of PI3K/Akt signaling, wortmannin inhibits proliferation and induces apoptosis in treated cells in a dose- and time-dependent manner [8]. Herein, we investigated the synergistic effect of caffeine and wortmannin toward gamma-radiation on HeLa cells in attempt to understand how these PIK3 inhibitors increase the effect of the DNA-damaging agent.

Materials and methods

Cell culture and treatment – Human HeLa cells (obtained from ATCC) were cultured in DMEM with 10% FBS, supplemented with antibiotics, in incubator at 37°C with 5% CO_2. Cells were treated with 20 mM caffeine or 20 μM wortmannin. Irradiation was carried out with ^{137}Cs λ-source.

Cell viability assay – The viability of HeLa cells was determined by MTT assay following manufacturer's instructions (Sigma Chemical Company). Briefly, cells (1×10^4 cells/well) were grown in 96-well cell culture plates and were incubated with 20 mM caffeine or 20 μM wortmannin or irradiated in 100 μl of complete culture medium with 10% FBS. After treatments, cells were incubated with 10 μl of MTT reagent for 4 h. The cell supernatants were removed and isopropanol was added to dissolve the formazan crystals. The absorbance of the formazan product was measured at 570 nm.

Cell cycle analysis was determined by fluorescence activated cell sorting (FACS) as described previously [9]. Cells were detached from tissue culture dishes by trypsinization, washed with PBS, and fixed in 70% ethanol for 12 hours at 4°C. After washing with PBS, the cells were incubated with 50 μg/ml of RNase A for 30 min at 37°C and stained with 20 mg/ml propidium iodide at room temperature for 90 min. 1×10^4 cells/sample were analysed with a Becton Dickinson (FACSCalibur) cell sorter, using CellQuest software (Becton Dickinson).

Results and discussion

To examine radiosensitizing effect of PI3K inhibitors we irradiated exponentially growing HeLa cells with 10 Gy gamma-radiation and cultured them for 3 days. Before irradiation, part of the cells was pretreated for 1 hour with 2 mM caffeine or 20 μM wortmannin. Third aliquot of cells was treated either with caffeine or wortmannin immediately after irradiation for 16 hours. The viability of HeLa cells was measured by MTT assay. We determined that 72 hours after irradiation with 10 Gy, cell viability was significantly decreased. The co-treatment with PI3K inhibitor before or immediately after irradiation increases the cell killing effect of gamma-radiation at all time points with the both inhibitors (Figures 1A and 2A).

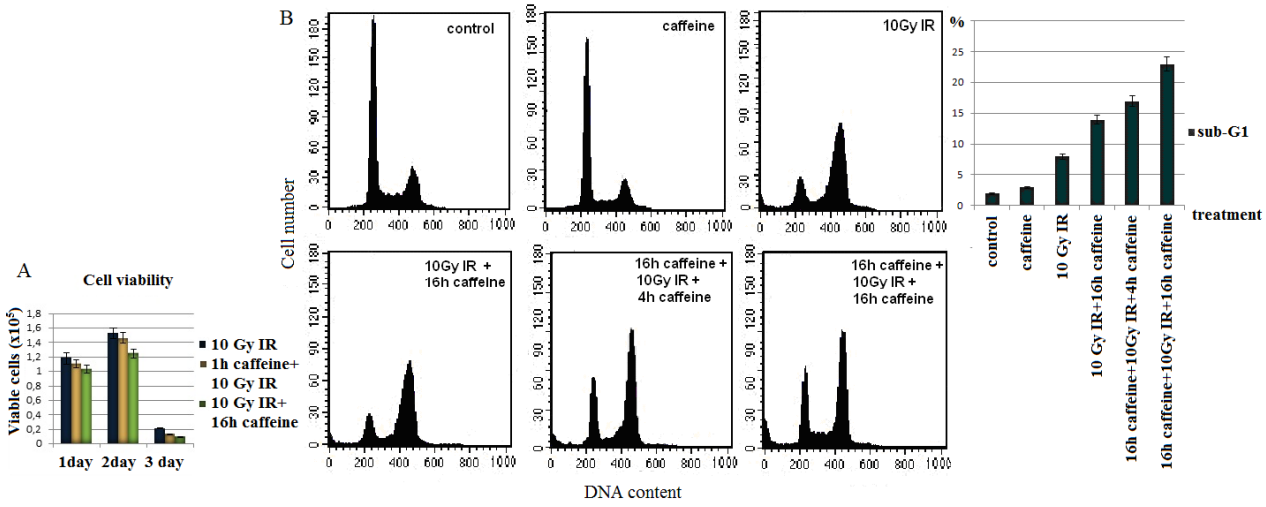

Figure 1. A) Cell viability of HeLa cells treated with 2mM caffeine and/or 10 Gy gamma-radiation. B) Flow cytometry analysis of HeLa treated with 2mM caffeine and/or 10 Gy gamma-radiation.

In agreement with the data from the literature [4] as well as in correlation with our previously published results [10], the flow cytometric analysis showed that as a consequence of radiation-induced DNA damage the treated cells activated G2/M phase cell cycle arrest by 18 h after irradiation (Figures 1B and 2B). The presence of 2 mM caffeine partially abrogated gamma-radiation-imposed G2/M phase arrest in a time-dependent manner (Figure 1B). The addition of the inhibitor after irradiation had a slight effect on the cell cycle distribution but if it was added before and persisted during and after irradiation, the abrogation of the G2/M phase arrest was stronger and time-dependent. The abrogation of the cell cycle arrest by caffeine drives cells to undergo mitosis with unrepaired DNA, leading to apoptosis, indicated by increased sub-G1 phase fraction by the flow cytometric analysis (Figure 1B).

The other PI3K inhibitor wortmannin also showed the same mechanism of radiosensitization - through abrogation of the radiation-induced G2/M phase arrest. The addition of wortmannin before and its persistence during and after irradiation increased its radiosensitizing effect. It is shown in Figure 2B, that after irradiation and in the presence of wortmannin, the number of cells which have undergone mitosis was increased (indicated by the decreased G2/M phase fraction). The number of cells which have undergone apoptosis was also increased (indicated by the appearance of sub-G1 fraction).

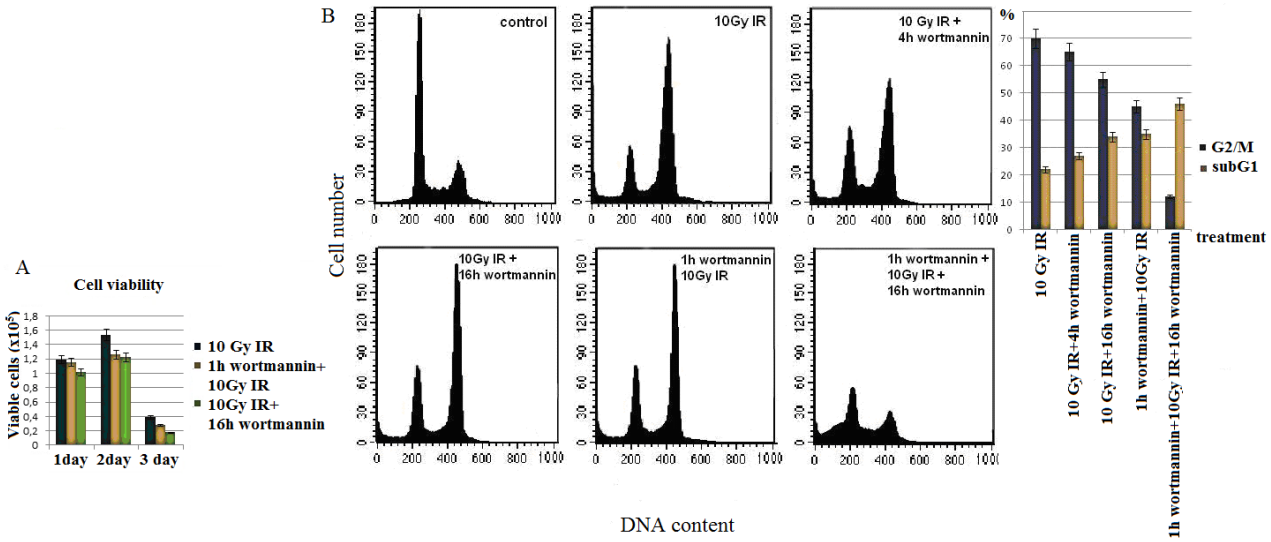

Figure 2. A) Cell viability of HeLa cells treated with 20 μM wortmannin and/or 10 Gy gamma-radiation. B) Flow cytometry analysis of HeLa treated with 20 μM wortmannin and/or 10 Gy gamma-radiation.

Cells respond to DNA damage by activating a network of signal transduction pathways including cell cycle checkpoints, DNA repair and damage tolerance pathways. In the DNA damage response, sensors, transducers and effectors are coordinated and regulate DNA repair with the cell cycle progression in order to ensure genome integrity. Some important components of these cascades belong to the PI-3 kinase-related kinase family. PI3K inhibitor caffeine has been shown to inhibit various isoforms of PI-3 kinase, components of the PI-3 kinase signaling pathway and its downstream targets, including ATM, ATR, DNA-PK, Chk1, p53, Akt, and mTOR [6]. Wortmannin, which is also a nonspecific inhibitor, is able to react with DNA-PKCs, ATM [11], ATR, PI4K, mTOR [12] and Akt [8]. Inhibition of one or more components of the cascades can abrogate the radiation-induced G2/M arrest and drive cells to apoptosis. Hence, the combination of PI3K inhibitor and gamma-radiation act synergistically to reduce cervical cancer cell viability.

Conclusion

In the last years, there has been significant interest in PI3Ks as important molecular targets for anticancer therapy [13]. More than fifteen PI3K inhibitors are in various stages of clinical studies [7]. Some of them show a potential to be used as a single agent as well as in combination therapy. Considerable efforts have been made in finding new non-toxic PI3K inhibitors and in studying the mechanisms of their action both in vitro on different cancer cell types and in vivo. Our results showed that the presence of PI3K inhibitor caffeine or wortmannin partially abrogated DNA-damage-induced G2/M phase arrest and sensitized HeLa cells towards gamma-radiation. Therefore, the combination of DNA damaging agent and drugs targeting DNA-damage-response enzymes possesses an exciting potential as a new anticancer therapy.

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