Regulation of paclitaxel-induced programmed cell death by autophagic induction: A model for cervical cancer

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Objective
Autophagy plays a vital role in homeostasis by combining organelles and cellular proteins with lysosome under starvation conditions. In addition, autophagy provides tumor cells with a source of energy. Continued autophagy will induce cell death. Here we aim to see if autophagic induction has an effect on conventional chemotherapeutic agents.

Methods
Rapamycin, or mammalian target of rapamycin and paclitaxel, apoptosis-inducing agents were used autophagy in HeLa cervical cancer cells.

Results
Growth inhibition of cells was not observed after the application of 0, 10, 20 nM of paclitaxel with or without rapamycin. Using a 5 nM concentration of paclitaxel, rapamycin administration inhibited cell growth significantly compared to no treatment. This implies the synergic antitumor effect of paclitaxel and rapamycin. Paclitaxel itself did not show any autophagic effect on cells but did show cell apoptosis by flow cytometry. Light chain 3, a microtubule-associated protein, which reflect autophagy, was increased with 5 nM of paclitaxel after pretreatment with 10 nM of rapamycin.

Conclusion
These findings suggest that the autophagic inducer, rapamycin, can potentiate autophagic cell death when added as an apoptosis-inducing chemotherapeutic agent. In conclusion, the control of autophagy may be a future target for chemotherapy.

Keywords: Apoptosis; Autophagy; Cervical neoplasms; mTOR protein; Paclitaxel

Introduction
The autophagy maintains the homeostasis by binding the intracellular organelles and protein to lysosome and thereby degrading them when the nutrient supply to the cells is blocked. The major functions of autophagy include self-control, response to the stress and the alternative measures for energy and survival. But if this is persistently present, this would lead to a complete disruption of the cellular morphology. These phenomena are referred to as type II programmed cell death or intracytoplasmic cell death. It is
known that the cancer cells supply the nutrients through the autophagy but this leads to the apoptosis when exposed to the long-standing stimuli [1,2].

One of the anti-cancer drugs that are commonly used to treat patients with gynecological cancer, paclitaxel has an anti-cancer effect are based on the apoptosis [3]. But most of the patients with progressive or recurrent cancer have a lower rate of treatment response to these anti-cancer drugs. To overcome this, efforts have been made to raise the treatment response by administering concomitant anti-cancer drugs rather than single one and introducing new anti-cancer drugs. To date, these efforts have revealed the limitations to the applicability to a clinical setting.

On the other hand, it is known that the phosphatidylinositol 3 phosphate kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway as well as the apoptosis is involved in the mechanisms by which the autophagy occurs. As described here, if there are any chances that these two involved mechanisms for apoptosis might be regulated, this would further lead to the programmed cell death. In this study, we examined whether there is a possibility to maximize the treatment effect using rapamycin, an inducer of the autophagy, concomitantly with conventional types of apoptosis-based anti-cancer drugs in a model of uterine cervical cancer.

**Materials and Methods**

1. **Culture of HeLa cell lines and the treatment of them with drugs**

The HeLa uterine cervical cancer cell lines were cultured in a Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) in conditions of 37°C and 5% CO². 1 × 10⁴/mL HeLa cell lines were cultured on a 96-well plate (Nunc, Rochester, NY, USA) for 48 hours. This was followed by the treatment with an anti-cancer drug, paclitaxel (Sigma-Aldrich, St. Louis, MO, USA), and rapamycin (Sigma-Aldrich), an inducer of autophagy.

Following the culture of HeLa uterine cervical cancer cell lines, they were treated with paclitaxel at varying concentrations of 0, 5, 10, 15, and 20 nM for 24 hours. This was followed by the examination of their inhibitory effects on cell survival. The cell survival was examined 24 hours after the treatment with rapamycin at varying concentrations of 0, 5, 10, 15, and 20 nM.

On the other hand, to examine the synergistic effects of two drugs in increasing the survival of cells, we administered paclitaxel at varying concentrations of 0, 5, 10, and 20 nM to the HeLa cells that had been pre-treated with rapamycin 10 nM for three hours. Then, the cell viability was examined.

The assessment was done with the methods for measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Colorimetric assay kit, Chemicon Inc., Temecula, CA, USA) which had been modified from tetrazolium based colorimetric assay. Following the addition of MTT samples 20 mg/mL, the culture was done for four hours. After the removal of culture medium, followed by the addition of dimethylsulfoxide (DMSO), an analysis was done at a wavelength of 450 nm.

2. **The quantitative analysis of the apoptosis and autophagy due to paclitaxel in HeLa uterine cervical cancer cell lines**

1-5 × 10⁵ HeLa uterine cervical cancer cells were cultured for a day and then treated with 5 nM paclitaxel. Twenty-four hours after the treatment with paclitaxel, both paclitaxel-treated cells and their non-treated controls were administered trypsin. Then, the cells were placed in phosphate buffered saline (PBS) containing 1% paraformaldehyde. This was followed by the fixation with 70% cool ethanol. Annexin V propidium iodide (PI) staining were done with ApoDETECT Annexin V-FITC kit (Invitrogen, Calsbad, CA, USA). According to the manufacturer’s instructions, changes in the apoptosis were examined. The stained cells were subjected to a quantitative apoptosis assay using a flow cytometry (FACS Vantage SE, BD Bioscience, San Jose, CA, USA). Thus, the cells undergoing apoptosis were counted.

For the quantitative analysis of autophagy, changes in the development of acidic vesicular organelles were examined. That is, 5 × 10⁵ HeLa cells were cultured overnight. This was followed by a 24-hour treatment with 5 nM paclitaxel. Paclitaxel-treated cells and their non-treated controls were stained with acridine orange (Sigma-Aldrich) at a concentration of 0.5 ug/mL at 37°C for 15 minutes with the sunlight blocked. The cells were rinsed with PBS twice and then treated with trypsin. Then, the cells were suspended on PBS containing 1% FBS, whose number was counted using a flow cytometry. This was followed by the analysis using CelQuest 7.0 software (Beckman Coulter Co., Brea, CA, USA).