学 位 論 文 の 要 旨

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学　位　論　文　名　Protective Roles of Cytoplasmic $p21^{Cip1/Waf1}$ in Senolysis and Ferroptosis of Lung Cancer Cells

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論 文 内 容 の 要 旨

INTRODUCTION

When cancer cells are treated with anticancer drugs, some of them mitigate DNA damage by stopping or slowing down their cell cycle, a process known as cellular senescence. Recently, some studies have shown that therapy-induced senescent (TIS) cancer cells produce several cytokines and factors, known as a senescence-associated secretary phenotype (SASP), which promote recurrence and metastasis of residual cancer cells. Therefore, TIS has been considered a major hurdle that must be overcome to improve the prognosis of cancer patients receiving anticancer therapies.

The cyclin-dependent kinase (CDK) inhibitors $p16^{Ink4a}$ and $p21^{Cip1/Waf1}$ are two key molecules involved in cellular senescence. TIS is accompanied by increasing expression of these CDK inhibitors. In terms of human adenocarcinoma, inactivation of the *p16* gene is often observed, suggesting that p21 plays a crucial role in the survival of TIS cancer cells. In addition, p21 is involved in various cell functions and can work as a tumor suppressor as well as a tumor promoter. Despite its role in promoting apoptosis, p21 prevents apoptosis in cancer cells in response to anticancer therapies.

A new approach of selectively removing senescent cells known as senolysis has been proposed. Senolytic drugs can preferentially induce cell death in senescent cancer cells. ABT-263 (navitoclax) is one such drug that was identified as an inhibitor against Bcl-2, Bcl-xL, and Bcl-w. In this study, we examined the senolytic effects of ABT-263, as well as its analogue ABT-737, on TIS human lung adenocarcinoma cells. In addition, we found that pemetrexed (PEM) alone induced ferroptosis, a new type of cell death, in lung adenocarcinoma cells.

MATERIALS AND METHODS

Two human lung adenocarcinoma cell lines, A549 and PC9, were used. Cell death was assessed using the annexin V-FITC Apoptosis Detection Kit and PI. For immunoblot assay, the following antibodies were used: anti-$p21^{Cip1/Waf1}$, anti-$p16^{Ink4a}$, anti-p53, anti-phospho-p53, anti-glutathione peroxidase 4 (GPx4) antibody, anti-TATA-binding protein (TBP), anti-GAPDH, and anti-β actin. Intracellular ROS were measured using carboxy-H_2DCFDA. Lipid peroxidation was measured using Liperfluo. P21-knockout A549 (A549-KO) cells were established by the CRISPR/Cas9 method. To establish p21-reexpressing cells from A549-KO cells (A549-RE), pEB Multi-Neo vector encoding a human *p21* gene was transfected into A549-KO cells. P21-overexpresing A549 (A549-OE) cells were established by transfection with the p21-encoding pEB Multi-Neo vector. In *in vivo* xenograft models, female BALB nude mice were injected subcutaneously with A549 (2×10^6) cells with Matrigel into the right flank. When the tumor diameter was approximately 5–6 mm, the mice were divided into four groups. PEM (100 mg/kg) was intraperitoneally (i.p.) injected on days 0, 1, 3, 4, 6, 7, 9, and 10 after grouping. On days 2, 5, 8, and 11 after grouping, cancer-bearing mice were administered i.p. with ABT-737 (50 mg/kg). The tumor volume (mm^3) was calculated as follows: tumor volume = (length × $width^2$) ÷ 2. All experiments with animals in this study were approved by the Animal Care and Use Committee of Shimane University (IZ3-107). In utilizing clinical database, the Kaplan-Meier plotter was used for univariate analysis of survival time according to *CDKN1A* gene expression in lung cancer patients. To analyze parametric and nonparametric data, Student's *t*-test (two groups) and analysis of variance (ANOVA) with the Tukey-Kramer test (more than two groups) were used. A P value < 0.05 was chosen to indicate statistical significance.

RESULTS AND DISCUSSION

First, we examined the effects of doxorubicin (DXR) on the expression of p21, p16 and p53 in A549 and PC9 cells. Immunoblots showed that DXR significantly increased cytoplasmic p21 expression only in A549 cells. P16 was expressed in PC9 cells but absent in A549 cells. Next, we examined the effects of ABT-263, a senolytic drug, on A549 and A549-KO cells. Pretreatment with DXR increased the sensitivity of A549 cells to ABT-263 or a Bcl-xL inhibitor A1331852, however, this effect was not observed after culture with Blc-2 inhibitor ABT-199. ABT-263 increased the percentage of annexin V^+ cells in DXR-pretreated parental A549 cells, which was significantly inhibited by pan-caspase inhibitor z-VAD. Furthermore, the percentage of annexin V^+ cells was higher in A549-KO cells than in parental A549 cells.

PEM increased the expression of cytoplasmic p21 in A549 cells. A combination of PEM and ABT-737 significantly increased the percentage of annexin V^+ A549 cells. In a xenograft

model, combining PEM and ABT-737 suppressed the *in vivo* growth of A549 cells in nude mice.

During the *in vitro* culture of A549-KO cells with PEM alone, we observed drastic cell death after 4 days of culture. PEM treatment started to increase the percentage of annexin V^+ A549 and A549-KO cells on day 2 after the initiation of culture, and annexin V^+ cells continued to increase thereafter only in the PEM-treated A549-KO group. Adding ferrostatin-1, a ferroptosis inhibitor, significantly decreased the percentages of both PI^+ cells and annexin V^+ cells. In addition, higher levels of ROS were observed in PEM-treated A549-KO cells. The *in vivo* growth of A549-KO in nude mice was significantly faster than that of parental A549 in untreated nude mice. When these tumor-bearing mice were treated with PEM, significant growth suppression was observed only in A549-KO-grafted mice.

We finally investigated whether a difference in *CDKN1A* expression had any influence on the prognosis of lung cancer patients. Based on mRNA levels, patients with lung adenocarcinoma with high *CDKN1A* expression showed significantly poorer overall survival and progression-free survival compared to those with low *CDKN1A* expression.

Senolysis in DXR-treated A549 cells was induced by ABT-263. In addition, senolysis was mainly caused by caspase-dependent apoptosis. Given that TIS cells increase cytoplasmic p21 expression, p21 may have inhibitory effects on senolysis. We also utilized PEM, an anti-folate drug, which has been widely used for the treatment of NSCLC patients and can induce senescence in NSCLC cell lines. In addition, ABT-737 preferentially induced cell death in PEM-treated A549 cells *in vitro* and synergized with PEM *in vivo*.

In comparing the effects of PEM on A549 and A549-KO cells, we observed drastic cell death in A549-KO cells 2 days after the initiation of culture, and this cell death was not apoptosis but ferroptosis. Ferroptosis is a new type of cell death that depends upon Fe^{2+} and ROS, particularly lipid peroxidation. Intriguingly, a low level of ferroptosis was induced even in PEM-treated parental A549 cells in which the expression of cytoplasmic p21 was increased. Therefore, we speculated that PEM increased lipid peroxidation in both parental A549 and A549-KO cells, but that increased cytoplasmic p21 in senescent A549 cells hindered the induction of ROS, other than lipid peroxidation.

CONCLUSION

In conclusion, we show that senolytic drugs induce caspase-dependent apoptosis in TIS A549 cells, and that PEM alone induced ferroptosis, a new type of cell death. In addition, cytoplasmic p21, which was increased in TIS lung adenocarcinoma cells, plays protective roles in senolysis and ferroptosis.