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Synergistic anti-proliferative effects of mTOR and MEK inhibitors in high-grade chondrosarcoma cell line OUMS-27

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A B S T R A C T

Chondrosarcoma is a malignant bone tumor that produces cartilaginous neoplastic tissue. Owing to the absence of an effective adjuvant therapy, high-grade chondrosarcoma has a poor prognosis. Therefore, it is important to develop an effective adjuvant therapy to prevent the recurrence and metastasis. Mammalian target of rapamycin (mTOR), a central regulator of cell growth, metabolism, proliferation, and survival, is considered an important target for anticancer drug development. The mitogen activated protein kinase (MAPK) pathway is another highly implicated cellular pathway in cancer and is thought to have compensatory effects in response to the inhibition of the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling pathway. We investigated the mechanism of anti-proliferative effect of the mTOR inhibitor rapamycin and MAPK/ERK (MEK) inhibitor PD 0325901, and the combined effect of rapamycin and PD 0325901 on human chondrosarcoma cell line (OUMS-27). Combination therapy with rapamycin and PD 0325901 showed a stronger anti-proliferative effect on OUMS-27 cells than rapamycin monotherapy. We confirmed that the dual inhibition of the PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways had synergistic anti-proliferative effects in OUMS-27. Our results suggest that combination therapy of mTOR and MEK inhibitor could be an effective therapeutic approach against chondrosarcoma.

1. Introduction

Chondrosarcoma is a heterogeneous group of malignant bone tumors that produce cartilaginous neoplastic tissue (Chow, 2007; Gelderblom et al., 2008). It is the third most common primary malignancy of bone after myeloma and osteosarcoma, accounting for approximately 20% of all malignant bone tumors (Hogendoorn et al., 2013). Chondrosarcoma is relatively resistant to conventional chemotherapy and radiotherapy (Chow, 2007; Gelderblom et al., 2008; Bovée et al., 2010; Wu et al., 2012). Prognosis is strongly correlated with histological grade and the adequacy of surgery (Gelderblom et al., 2008; Bovée et al., 2010; Unni and Inwards, 2010). Surgical resection is an effective treatment for low-grade chondrosarcoma. However, the prognosis for high-grade chondrosarcoma remains poor, even for the cases of adequate surgical resection owing to the high incidence of local recurrence and metastasis to the lung (Bovée et al., 2010) and the absence of an effective adjuvant therapy (Yuan et al., 2005; Fong et al., 2007; Wu et al., 2012). Therefore, it is important to develop an effective adjuvant therapy to prevent the recurrence and metastasis of chondrosarcoma. Recently, some translational studies have predicted the effects of gene therapy using tyrosine kinase inhibitors, such as Src kinase inhibitor, aromatase inhibitors, and Bcl inhibitors (Cleton-Jansen et al., 2005; Chen et al., 2015; de Jong et al., 2016). Further improvements in the prognosis for high-grade chondrosarcoma are expected by adjuvant chemotherapy.

Mammalian target of rapamycin (mTOR), a protein kinase in the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling pathway, is the central regulator of cell growth, metabolism, proliferation, and survival. mTOR is activated by growth factors and their receptors *via* the PI3K/Akt/mTOR signaling pathway. Activated mTOR phosphorylates two major downstream proteins, S6 kinase 1 and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which regulate translation and cell growth (Bjornsti and Houghton, 2004; Dancey, 2006; Wullschleger et al., 2006). According to previous reports, the PI3K/Akt/mTOR signaling pathway is hyper-activated as a consequence of oncogenic transformation in many human malignancies (Bjornsti and Houghton, 2004; Dancey, 2006). In tumor cells, the inhibition of mTOR induces apoptosis and cell cycle arrest at the G1/S phase (Huang et al., 2001). These observations indicate that mTOR is an important therapeutic target for anticancer drug development. However, there are insufficient preclinical data regarding the role of the mTOR signaling pathway in chondrosarcoma (Bernstein-Molho et al., 2012).

The mitogen activated protein kinase (MAPK) pathway is also highly implicated in cancer development (Pitts et al., 2014). MAPK/

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ERK (MEK) complexes are members of the RAF/MEK/ERK signaling pathway. In many tumors, signaling through this pathway leads to cell proliferation and resistance to apoptosis (Hoshino et al., 1999; Mueller et al., 2000; Pitts et al., 2014). Previous reports in other tumor types have suggested that the activation of the RAF/MEK/ERK signaling pathway mediates resistance to PI3K inhibitors (Engelman et al., 2008; Renshaw et al., 2013). These findings indicate the existence of cross-talk between the PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways (Pitts et al., 2014). It is proposed that the RAF/MEK/ERK signaling pathway has compensatory effects with the inhibition of the PI3K/Akt/mTOR signaling pathway (Zitzmann et al., 2010). Recently, many reports have suggested the synergistic anti-tumor efficacy of the dual inhibition of both the PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways (Renshaw et al., 2013; Sheppard et al., 2013; Ewald et al., 2014; Pitts et al., 2014).

In this study, we investigated the influence of the RAF/MEK/ERK pathway on mTOR inhibition in human high-grade chondrosarcoma cell line, OUMS-27, which is widely used for studies of chondrosarcoma (Kunisada et al., 1998; Akyol et al., 2015; Lu et al., 2016). Furthermore, to explore the synergistic effects of the dual inhibition of both the PI3K/Akt/mTOR and RAF/MEK/ERK signaling pathways, we evaluated combination therapy using the mTOR inhibitor rapamycin along with the MEK inhibitor PD 0325901 on OUMS-27 cells.

2. Materials and methods

2.1. Cell culture

The OUMS-27 cell line was obtained from Okayama University and characterized by a short tandem repeat analysis (access code; CVCL_3090). Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Biowest, Nuaille, France) and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA) under an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were confirmed for the absence of mycoplasma infection using e-Myco Mycoplasma PCR Detection Kit (iNtRON Biotechnology, Gyeonggi-do, Korea).

2.2. Cell viability assay (MTT assay)

OUMS-27 cells were seeded on a 96-well plate (5×10^3 cells/well) and maintained for 24 h before treatment. Subsequently, they were incubated with culture medium alone as a control or with medium containing rapamycin (553210-5MG lot. D00168866, EMD Millipore Corporation, Billerica, MA, USA) or the MEK inhibitor PD 0325901 (AdooQ Bioscience, Irvine, CA, USA) at various concentrations, *i.e.*, 1, 10, 20, 30, and 40 μM. Combination therapy was examined using various concentrations of rapamycin (up to 40 μM) with a fixed concentration (10 μM) of the MEK inhibitor PD 0325901. Cell viability was measured using the Cell-Titer-Blue Cell Viability Assay (Promega Co., Madison, WI, USA) according to the manufacturer's instruction. After 48 h post rapamycin or dual inhibition treatment, MTT solution was added to each well followed by incubation at 37 °C for an additional 1 h. The optical densities of each well were measured at 490 nm using the SH-1000Lab microplate reader (CORONA ELECTRIC, Ibaraki, Japan).

2.3. Western blotting

After 48 h of treatment with 10 μM rapamycin, 10 μM PD 0325901, or a combination of 10 μM rapamycin and 10 μM PD 0325901, cells were harvested and proteins were extracted in 1 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 1% (w/v) SDS, 1 mM PMSF, 1% (w/v) sodium deoxycholate, and 0.5% (w/v) Protease Inhibitor Cocktail (Sigma Aldrich, St. Louis, USA). Protein concentration was determined in the supernatants using the Qubit® Protein Assay Kit (Molecular Probes, Eugene, OR, USA) with the Qubit® 2.0 fluorometer. Each aliquot was then loaded onto an 8% polyacrylamide gel. After electrophoresis, gels were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). To block the nonspecific binding, the membranes were incubated with 1% bovine serum albumin in tris-buffered saline (TBS) overnight, followed by incubation with primary antibodies against ERK (diluted 1000×; rabbit polyclonal antibody #9102 lot 19, Cell Signaling Technology, Danvers, MA, USA), phosphorylated (p)-ERK (diluted 1000×; rabbit polyclonal antibody #9101 lot 26, Cell Signaling Technology), and GAPDH (diluted 3200×; mouse monoclonal antibody #5G4, HyTest, Turku, Finland). After rinsing, membranes were probed with horseradish peroxidase (HRP)-linked anti-rabbit IgG (#7074 lot 17, Cell Signaling Technology) and anti-mouse IgG (#7076 lot 21, Cell Signaling Technology) secondary antibodies. The chemiluminescent reaction was performed using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK). Protein expression was detected using the LAS-3000 Lumino Image Analyzer (Fuji Photo Film, Tokyo, Japan). The signal intensities were further analyzed using Multi Gauge software (version 3.0; Fuji Photo Film). Each quantitative estimate was adjusted against the control.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

OUMS-27 cells were grown and treated with 10 μM rapamycin, 10 μM PD 0325901,or a combination of 10 μM rapamycin and 10 μM PD 0325901 for 48 h. Harvested cells were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using the Apoptosis Detection Kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. Immunoreactivities were observed using an optical microscope (BX53, Olympus Corporation, Japan).

2.5. Flow cytometric analysis for apoptosis

OUMS-27 cells (5×10^5), both adherent and suspended, were treated with various concentrations of rapamycin or PD 0325901, *i.e.*, 10 nM, 100 nM, 1 μM, and 10 μM, or medium alone (control) and harvested after 48 h. Cells were fixed in 1% (w/v) PFA in PBS (pH 7.4). The combination therapy was performed using various concentrations (up to 10 μM) of rapamycin with a fixed concentration of PD 0325901 (10 μM). Harvested cells were fixed in 1% PFA in PBS. The APO-DIRECT™ Apoptosis Detection Kit (Becton-Dickinson, San Jose, CA, USA) was used according to the manufacturer's protocol. Apoptotic cells were analyzed by FACScan flow cytometry and BD Diva Software Version 4.1 (Becton-Dickinson).

2.6. Caspase activity

OUMS-27 cells were plated at a cell density of 1×10^4 cells/well on a 96-well plate 24 h before treatment. After 12 h treatment with 10 μM rapamycin, 10 μM PD 0325901, or medium alone, cell viability was measured using the CellTiter-Blue Cell Viability Assay (Promega). The combination therapy was performed using 10 μM rapamycin with 10 μM PD 0325901. The activities of caspase-3/7, caspase-8, and caspase-9 were measured using the luminescence-based Caspase-Glo™ Assay (Promega). Fluorescent intensities for cell viability and luminescent intensities for caspase activity were measured using the GloMax-Multi Detection System (Promega). The activity of each caspase was adjusted against the corresponding cell viability as described previously (Shibata et al., 2007).

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