Inhibition of induced autophagy increases apoptosis of Nara-H cells

OSAMU NAKAMURA1, TOSHIKI HITORA1, TOSHIHIRO AKISUE2, TERUYA KAWAMOTO2, YOSHIKI YAMAGAMI1 and TETSUJI YAMAMOTO1

1Department of Orthopaedic Surgery, Kagawa University School of Medicine, Miki-cho, Kita-gun, Kagawa; 2Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan

Received June 9, 2011; Accepted July 15, 2011

DOI: 10.3892/ijo.2011.1148

Abstract. Inhibition of the mTOR signaling pathway promotes initiation of autophagy. However, recent studies indicate that autophagy is a self-defense mechanism of cancer cells that are subjected to anti-tumor agents and that blocking autophagy can trigger apoptosis. Here, we examined the effects of an mTOR inhibitor, temsirolimus, on a malignant fibrous histiocytoma (MFH) cell line, Nara-H cells, and the effect of suppressing autophagy on the induction of apoptosis in these MFH cells. In Nara-H cells, we examined the effects of temsirolimus treatment on cell proliferation using the CellTiter 96 AQueous One Solution Cell Proliferation Assay and on phosphorylation of mTOR pathway components and autophagy using Western blot-based assays. Furthermore, we examined the effects of temsirolimus with or without 3-methyladenine (3-MA) on induction of apoptosis using fluorescent microscopic analysis. In Nara-H cells, temsirolimus treatment inhibited cell proliferation, suppressed phosphorylation of mTOR pathway components, and induced autophagy as assessed by LC-3 II expression. Moreover, treatment with a combination of temsirolimus and 3-MA induced apoptosis in Nara-H cells. Apparently, simultaneous inhibition of autophagy and mTOR induced apoptosis in Nara-H cells because inhibition of autophagy prevented the cells from protecting themselves from the effects of the inhibition of mTOR. Therefore, a combination therapy that includes an mTOR inhibitor and an autophagy inhibitor (temsirolimus and 3-MA, respectively) may effectively treat MFH by inducing apoptosis in tumor cells.

Introduction

Malignant fibrous histiocytoma (MFH) is one of the most common soft tissue sarcomas in human adults (1). Advances in treatment of MFH have led to multimodal treatments that may include surgery, chemotherapy, and/or radiation therapy. These treatments have resulted in significant improvements in quality of life for patients with soft tissue sarcomas. However, the present chemotherapeutic protocols for some of these sarcoma are not as effective as those for other malignancies, and the prognosis for some patients with sarcoma is poor due to local tumor recurrence and metastases (2). Developing new strategies for assessing prognosis, overcoming resistance to anticancer therapies, and preventing or treating metastasis are important goals in the treatment of patients with sarcoma (3).

In recent years, drugs that target specific molecules have been developed as treatments for other human malignancies, including cancers and sarcoma. These drugs often selectively inhibit specific molecules, such as growth factor receptors or intracellular signaling proteins that are related to tumor proliferation, migration, and/or metastasis. Here, we focused on the mammalian target of rapamycin (mTOR) signaling pathway.

The mTOR protein is an essential serine/threonine kinase that belongs to the phosphoinositide-3-OH kinase (PI3K)-related kinase family (4). It is an intracellular protein that mediates cell growth, proliferation, migration, and survival and promotes angiogenesis in many cancer types (5,6). Inhibitors of mTOR have antitumor effects in many human malignancies (7,8). Inhibition of mTOR affects pathway-mediated transcription and translation, leading to cell cycle arrest and anti-angiogenesis. Regarding the anti-tumor mechanism of mTOR inhibitors, some studies indicate that inhibitors of Akt and its downstream target, mTOR signaling, have anti-tumor effects because inhibition of this pathway contributes to the initiation of autophagy (9-11).

Autophagy has recently gained attention because of its paradoxical roles in cell survival and cell death, particularly in the pathogenesis and treatment of cancer (12). Regulation of autophagy is highly complex with inputs from the cellular environment through the PI3K/Akt/mTOR pathway (13). Not surprisingly, there is an intricate relationship between autophagy and apoptosis. Recent studies indicate that autophagy can function as a self-defense mechanism in cells that are subjected to anti-tumor agents and that blocking autophagy can trigger activation of apoptosis (14-16). Based on these finding, it has been suggested that inhibitors of autophagy, such as 3-methyladenine (3-MA), might be an effective treatment for MFH because they activate apoptosis.

Recent studies demonstrate that 3-MA, a commonly-used inhibitor of autophagy, promotes chemotherapeutic drug-induced apoptosis in cancer cells. Reportedly, 3-MA inhibits the activity of PI3K and blocks the formation of pre-autophagosomes, autophagosomes, and autophagic vacuoles (17).
The purpose of this study was to examine the effects of the mTOR inhibitor, temsirolimus, on Nara-H cells (an MFH-derived cell line). We examined whether temsirolimus affected suppression of phosphorylation of proteins in the mTOR pathway and/or induction of autophagy in Nara-H cells. Furthermore, we examined whether a combination of temsirolimus and 3-MA induced apoptosis in Nara-H cells.

Materials and methods

Chemical reagents. Temsirolimus (CCI-779) was purchased from BioVision Research (Linda Vista, Mountain View, CA, USA), dissolved in dimethyl sulfoxide (DMSO), and stored at -20°C. The inhibitor of autophagy, 3-MA, was purchased from Sigma Chemical Co. (St. Louis, MO, USA), dissolved in dimethylformamid (DMF; 1 mg/ml), and stored at room temperature.

Cell lines and cell culture. Nara-H cells were purchased from ScienStuff Co. (Nara, Japan). The Nara-H cell line was established from a myxoid MFH of the uterus by Kiyozuka et al. (18). These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 100 U/ml penicillin. These cells were routinely maintained at 37°C in a humidified 5% CO2 atmosphere, and cultures were used at mid-log phase. Cells were divided into four groups, control (no inhibitor), tem (temsirolimus), 3-MA, tem plus 3-MA (temsirolimus + 3-MA), for the autophagy and apoptosis assays. Cells were seeded onto culture dishes and cultured in growth medium for 48 h, and then the growth medium was replaced with fresh medium with or without inhibitors.

In vitro proliferation assay. Cell proliferation was determined by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Cells were trypsinized and seeded at a density of 1x104 cells/well in 96-well cell culture plates with 200 µl culture medium containing 10% FBS and incubated for 48 h. Following this initial incubation, the growth medium was replaced with medium containing 10% FBS and temsirolimus at a concentration of 0, 1, 2.5, 5, 10, 25, 50, or 100 µM. After 24, 36 or 48 h, the medium was removed, the cells were washed with phosphate-buffered saline (PBS), fresh medium containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (100 µl medium plus 20 µl MTS reagent/well) was added to each well. In the experiments testing the combined effect of temsirolimus and 3-MA, cells were pretreated with 5 mM 3-MA for 90 min before temsirolimus was added to the culture medium, and then cells were treated with 25 µM temsirolimus with 5 mM 3-MA for 24 h. In experiments testing the effect of temsirolimus or 3-MA, cells were treated with 35 µM temsirolimus or 5 mM 3-MA for 24 h. Following treatment, the culture medium was replaced with lysis buffer (Cell Signalling Technology, Beverly, MA, USA), and cells were lysed on ice for 20 min. The cell lysates were spun at 15,000 x g using the Tomy MTX-150 centrifuge (Tomy Seiko Co., Ltd., Fukuoku, Japan) at 4°C for 30 min. The supernatant was then collected as the total cellular protein extracts. The protein concentrations were determined by Protein Assay Bicinchoninate kit (Nacalai Tesque, Inc., Kyoto, Japan) and standardized with bovine serum albumin. The samples of total cellular protein were loaded onto an SDS polyacrylamide gel (7.5, 10, or 12% commercial precast gel, Wako, Tokyo, Japan), and the proteins were separated by SDS-PAGE under reducing conditions. The mTOR and phospho-mTOR proteins were separated on a 7.5% SDS gel; the Akt, phospho-Akt, p70 ribosomal protein S6 kinase (p70S6K), phospho-p70S6K, α-tubulin, poly-(ADP-ribose) polymerase (PARP) proteins were separated on 10% SDS gel; and 4-E-binding protein 1 (4E-BP1), phospho-4E-BP1, and microtubule-associated protein light-chain 3 (LC-3) proteins were separated on a 12% SDS gel. The separated proteins were electrophoretically transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The membranes were blocked for 90 min in the blocking buffer containing Tris-buffered saline (TBS-T) and 10% EZ block (Atto Co., Tokyo, Japan). Then, the membranes were incubated with primary antibodies which diluted in the blocking buffer overnight at 4°C. Antibodies for Akt, phospho-Akt (Thr 308) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mTOR and anti-phospho-mTOR (Ser 2448) antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Anti-4E-BP1, anti-phospho-4E-BP1 (Ser 65), anti-p70S6K, and anti-phospho-p70S6K (Thr421/Ser424) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA), and anti-α-tubulin antibody was purchased from Sigma. Anti-PARP antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-LC-3 antibody was purchased from MBL Co. (Nagoya, Japan). These primary antibodies are listed and characterized in Table I.

The specific HRP-conjugated secondary antibody incubations were performed overnight at 4°C with gentle agitation. Bound antibodies were detected using the ECL plus Western blotting detection system (GE Healthcare Bio-Sciences) and LAS-1000 plus imaging analyzer (Fuji Film Co., Tokyo, Japan). Specific signals were quantified by densitometric analysis (Image J software).

Cell cycle fraction by flow cytometry. Cells were stained with propidium iodide (PI) to assess the cell cycle phase during fraction by flow cytometry. Cells were seeded at a density of 6x10^5 and cultured for 48 h. In the experiments testing the combined effect of temsirolimus and 3-MA, cells were pretreated with 5 mM 3-MA for 90 min before temsirolimus was added to the
Tissue culture medium, and then cells were treated with 35 µM temsirolimus with 5 mM 3-MA for 24 h. In experiments testing the effect of temsirolimus or 3-MA, cells were treated with 35 µM temsirolimus or 5 mM 3-MA for 24 h. Then cells were washed gently three times in PBS, fixed with 70% ethanol, and stored at -30˚C. The next day, the samples were washed three times in PBS and incubated with 10 µM RNase, DNase-free (Roche Applied Science, Penzberg, Germany) and 50 µM PI (Miltenyi Biotec Inc., Auburn, CA, USA) for 20 min at 37˚C. Cell-cycle analysis was performed using a Flow Cytometer (FC-500, Beckman Coulter, Inc., Brea, CA, USA) for evaluation of the sub-G fraction. Data are representative of 3 independent experiments.

**Determination of apoptosis by Annexin V-FITC and PI stain analysis.** Cells were trypsinized and seeded at a density of 6x10⁵ cells/well in 6-well cell culture plates in 2 ml culture medium with 10% FBS and then cultured for 48 h. In the experiments testing the combined effect of temsirolimus and 3-MA, cells were treated with 5 mM 3-MA for 90 min before temsirolimus was added to the culture medium, and then cells were treated with 35 µM temsirolimus with 5 mM 3-MA for 24 h. In experiments testing the effect of temsirolimus or 3-MA, cells were treated with 35 µM temsirolimus or 5 mM 3-MA for 24 h. Cells were then incubated with MDC for 15 min and immediately analyzed by fluorescence microscope (DMI 4000 B, Leica Microsystems, Wetzlar, Germany) using a fluorescence microscope equipped with a filter system (excitation wavelength of 460-500 nm, emission wavelength of 512-542 nm). Bright-field and fluorescence images were merged. To quantify MDC incorporation, at least 100 cells from each treatment group were chosen randomly to avoid bias.

**Statistical analysis.** All data and results presented are representative of, or calculated from, at least three independent experiments. For the cell proliferation assay, flow cytometry, and morphological examination, differences between treatment groups were determined with the StatView for Windows software package. The data collected in three independent experiments for each group are expressed as mean ± standard deviation (SD) and analyzed statistically using ANOVA with Fisher's PLSD post hoc test. A p<0.05 was regarded as statistically significant.

**Results**

**Temirolimus inhibited cell proliferation.** We examined the effect of temsirolimus on Nara-H cell proliferation using the CellTiter 96 AQueous One Solution Cell Proliferation Assay to determine whether temsirolimus inhibited cell proliferation. Temsirolimus inhibited Nara-H cell proliferation in a dose- and time-dependent manner. The IC₅₀ for 48 h of temsirolimus treatment in Nara-H cells was 25.86±2.36 µM (Fig. 1A).

We then examined the effect of temsirolimus with or without 3-MA on Nara-H cell proliferation. Based on the IC₅₀ of temsirolimus after 48 h, we examine proliferation of Nara-H cells treated with 25 µM Temsirolimus (tem group), 5 mM 3-MA (3-MA

<table>
<thead>
<tr>
<th>Target</th>
<th>Source</th>
<th>Host</th>
<th>Dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>St. Cruz Biot.</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>Phospho-Akt</td>
<td>St. Cruz Biot.</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>nTOR</td>
<td>R&amp;D Systems</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>Phospho-nTOR</td>
<td>R&amp;D Systems</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Cell Signalings</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>Phospho-4E-BP1</td>
<td>Cell Signalings</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>p70S6</td>
<td>Cell Signalings</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>Phospho-p70S6</td>
<td>Cell Signalings</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>PARP</td>
<td>BD Biosciences</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Anti-mouse</td>
</tr>
<tr>
<td>LC-3</td>
<td>MBL</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Anti-rabbit</td>
</tr>
<tr>
<td>α-tublin</td>
<td>Sigma</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Anti-mouse</td>
</tr>
</tbody>
</table>

*Table I. Primary antibodies used in Western blot analysis.*
group), or 25 µM temsirolimus and 5 mM 3-MA (tem-plus-3-MA group) for 48 h. Cell proliferation was significantly lower in the tem-plus-3-MA group than in the tem group (p<0.05) (Fig. 1B). These results indicated that 3-MA enhanced temsirolimus-mediated suppression of Nara-H cell proliferation.

**Inhibitory effects on the expression of Akt/mTOR pathway.** Western blot analyses demonstrated that temsirolimus and temsirolimus-plus-3-MA treatments promoted phosphorylation of key components in the Akt-mTOR pathway. We examined the time course of 4E-BP1, phospho-4E-BP1, and LC-3 expression in treated and control Nara-H cells. These proteins are downstream of mTOR, and LC-3, LC-3II (16 kDa) in particular, is the first protein to specifically localize to autophagosome membranes. Therefore, LC-3II expression can be used as a marker for the induction and initiation of autophagy. Over time, the levels of phosphorylated 4E-BP1 became lower in temsirolimus-treated Nara-H cells than in control cells, but there was no difference in total 4E-BP1 protein levels between the groups at any time-point. Based on the result of this time-course analysis, we concluded that a temsirolimus treatment of 90 min was sufficient for all further experiments. Moreover, this time-course analysis demonstrated that autophagy, as determined by LC-3II expression, was induced within 30 min of adding temsirolimus (Fig. 2A).

We also examined Akt, phospho-Akt, mTOR, phospho-mTOR, 4E-BP1, phospho-4E-BP1, p70S6K, phospho-p70S6K, and LC-3 expression in Nara-H cells exposed to different concentration of temsirolimus (ranging from 1 to 50 µM) for 90 min (Fig. 2B). Temsirolimus did not cause any change in the levels of phospho-Akt and phospho-mTOR. However, temsirolimus treatments resulted in dose-dependent decreases in both phospho-4E BP1 and phospho-p70S6K and p7OS6K, like 4EBP1, is a downstream effector of mTOR. These findings indicate that temsirolimus affects the mTOR pathway by
effectors by inhibiting phosphorylation of downstream effectors of mTOR. Moreover, temsirolimus treatments resulted in dose-dependent increases in LC-3II expression in Nara-H cells.

Finally, we examined PARP and LC-3 expression in Nara-H cells treated with 35 μM temsirolimus with or without 5 mM 3-MA and in untreated control cells (Fig. 2C). In cells treated with temsirolimus and temsirolimus plus 3-MA treated cells, PARP was cleaved from the inactive 116 kDa form to the active 89 kDa form. The expression of LC-3II was clearly higher in the group treated with 35 μM temsirolimus and 3-MA treatment than in the other groups (p<0.05).

**Discussion**

Soft tissue sarcomas, especially high-grade sarcoma like MFH, are clinically aggressive and frequently metastasize to various organs. Although many chemotherapeutic protocols, including doxorubicin, cisplatin and ifosfamide, are used for human sarcomas, protocols for sarcomas are often ineffective, and the prognosis for some patients can be extremely poor due to local recurrence and/or metastasis (2). In the absence of effective systemic chemotherapy treatments for aggressive sarcomas, targeted therapies are being investigated and used as treatments. Targeted drugs, including mTOR inhibitors, are currently tested as single agents or in combination with other agents for the treatment of sarcomas (6,20). Reportedly, blocking the mTOR pathway has implications for treatment of many diverse diseases (21).
mTOR, and this pathway plays a central role in cell survival and proliferation in many cancers (21,23,24). Abnormal activation of mTOR results in continuous activation of downstream transcription factors leading to malignant transformation. Unlike other kinases, mTOR is apparently genetically stable; it has not been shown to mutate in human malignancies. Therefore, mTOR represents a promising target for cancer therapy (6).

Once activated, mTOR phosphorylates translation-regulating factors p70S6K, a regulator of eukaryote translation, to increase the synthesis of key proteins that stimulate cell growth, proliferation, and survival. Active p70S6K and active 4E-BP1 are required for and independently mediate mTOR-dependent G1-phase progression (25). These observations indicate that mTOR inhibitors may suppress the phosphorylation of the downstream effectors, 4E-BP1 and p70S6K, and, therefore, delay progression of the cell cycle from G (1) phase to S phase. Our data demonstrated that temsirolimus did not cause any change in phospho-Akt and phospho-mTOR protein levels. However, temsirolimus treatments resulted in dose-dependent decreases in both phospho-4E-BP1 and phospho-p70S6K, two downstream targets of mTOR.

Inhibitors of mTOR can function as inhibitors of cell proliferation that leads to cell cycle arrest or as cytotoxic agents inducing apoptosis, depending on tumor cell type (26). In these cytotoxic actions, it is important that rapamycin induces apoptosis in certain cell systems (22). Rapamycin induces apoptosis only in cells lacking functional p53, and Huang et al have shown that their results may be useful in the conceptual development of tumor-selective therapy with rapamycin analogs or other inhibitors of mTOR (27). However, some studies show that mTOR
inhibitors induce cell death in some malignancies by autophagy rather than apoptosis (10,28). Recent studies also indicate that inhibition of the Akt/mTOR pathway is consistently associated with triggering autophagy in cancer cells (9). Moreover, other studies have shown that autophagy inhibitors, such as 3-MA, induced apoptosis in some cancer cell lines when used as an anti-cancer agent in combination with an mTOR inhibitor (14,29).

3-MA is an inhibitor of autophagy. However, recent reports indicate that 3-MA works well with chemotherapeutic drugs by triggering apoptosis in some cancer cells (29). In our experiment, we observed that using temsirolimus and 3-MA in combination induced more cell death in Nara-H cells than using temsirolimus alone. Therefore, it appears that together temsirolimus and 3-MA induced apoptotic cell death in Nara-H cells by inhibiting autophagy. Interestingly, our Western blots showed that LC-3II expression was higher in cells treated with a combination of temsirolimus and 3-MA than in other groups, but our fluorescence microscopy analysis of MDC-stained samples showed that there were fewer or smaller mature autophagosomes in cells treated with the temsirolimus and 3-MA combination than in other groups. Some authors report that in certain cases, though 3-MA prevents autophagy, 3-MA maintains LC-3 in its LC-3II form (30). Therefore, we suggest that while temsirolimus treatment led to LC-3II expression in Nara-H cells, the combined action of temsirolimus and 3-MA prevented actual autophagy in these cells.

Autophagy is a process in which subcellular membranes undergo dynamic morphological change (autophagosomes form and fuse with lysosomes) that lead to the degradation of cellular proteins and cytoplasmic organelles. Autophagy plays a protective role when cells encounter environmental stressors, such as starvation or pathogen infection (31,32). Autophagy also occurs under pathological conditions, such as neurodegenerative disease or heredity myopathies. Recently, increasing evidence has indicated that autophagy often plays a role in malignant diseases. Specifically, autophagy is believed to have an important role in tumor development. During the early stages of tumor formation, autophagy functions as tumor suppressor, and autophagic activity is often impaired in cancer cells.

In conclusion, temsirolimus had an inhibitory effect on cell proliferation and decreased the phosphorylation of mTOR pathway components in Nara-H cells. Temsirolimus induced autophagy in Nara-H cells, but treatment with a combination of temsirolimus and 3-MA suppressed autophagy and induced apoptosis in these cells.

It was thought that the autophagy inhibitor suppressed a self-defense mechanism in tumor cells and induced apoptosis. Therefore, the combination of an mTOR inhibitor and an autophagy inhibitor (temsirolimus and 3-MA, respectively) may be an effective treatment for MFH because this combination effectively induces the apoptosis pathways.

Acknowledgments

The authors thank Mr. Kouichi Yube (Division of Research Instru-ment and Equipment, Kagawa University, School of Medecine) for his excellent technical assistance with flow cytometry.

References


