Antitumor Effect of Temsirolimus against Oral Squamous Cell Carcinoma Associated with Bone Destruction

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Abstract

The mammalian target of rapamycin (mTOR) is engaged in the molecular pathogenesis of oral squamous cell carcinoma, which frequently invades the maxilla or the mandible. However, the effects of a mTOR inhibitor on bone destruction associated with oral squamous cell carcinoma are still unclear. In this study, we investigated the antitumor effect of temsirolimus-mediated mTOR inhibition against advanced oral squamous cell carcinoma. Temsirolimus inhibited the proliferation and migration of HSC-2 oral squamous cell carcinoma cells in vitro and suppressed the growth of oral squamous cell carcinoma xenografts in vivo. Significantly, we clearly show that temsirolimus inhibited osteoclast formation both in vitro and in vivo. Reverse transcriptase-PCR analysis showed that temsirolimus decreased the mRNA expression of receptor activator for nuclear factor-κB ligand, known as an osteoclast differentiation factor in bone stromal ST2 cells. Moreover, temsirolimus normalized blood-free calcium concentration in mouse models for humoral hypercalcemia. These findings suggest that mTOR signaling is a potential target of oral squamous cell carcinoma associated with bone destruction, and hence we describe the efficacy of temsirolimus for the treatment of advanced oral squamous carcinoma.

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Introduction

The invasion of oral squamous cell carcinoma cells into the maxilla or the mandible is a common clinical problem (1, 2). However, bone resection for treatment leads to physical damage and has a major influence on the quality of life because of the postoperative disruption of speech and swallowing function. Other approaches for the treatment of advanced oral squamous carcinoma are being investigated. Recently, single treatment of mammalian target of rapamycin (mTOR) inhibitors or combination treatment of the inhibitors with other anticancer agents showed antitumor activity in numerous tumors, such as metastatic pancreatic tumors (3), recurrent/metastatic breast cancer (4), advanced renal cell carcinoma (5–7), and advanced gastric cancer (8). It has been reported that tumors with continuous Akt activation are more sensitive to the inhibition of mTOR (9–11), and Akt is frequently activated in oral squamous cell carcinoma (12). It is still unclear, however, as to how mTOR signaling participates in osteoclastic bone resorption by oral squamous cell carcinoma. In the present study, we analyzed the antitumor effect of temsirolimus against advanced oral squamous cell carcinoma and investigated how mTOR signaling involves in osteoclastic bone resorption associated with tumor invasion.

Materials and Methods

Cell lines and culture conditions

The human oral squamous cell carcinoma cells HSC-2 and SAS, newly obtained from the Human Science Research Resources Bank (Osaka, Japan), and the murine bone marrow stromal cells ST2 and the murine macrophage cells RAW264.7, newly obtained from the RIKEN BioResource center Cell Bank (Tsukuba, Japan), were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. All cell lines were cultured in 10% CO2 at 37°C.

Purification of osteoclast progenitors

Bone marrow cells were washed twice by centrifugation in 20 mL of cold buffer containing sterile PBS supplemented with 0.5% bovine serum albumin (Sigma) and 2 mmol/L EDTA (Sigma). The cell pellet was resuspended

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in 80 μL buffer per 10^7 cells, and the cells were magnetically labeled by adding 20 μL of anti-CD11b microbeads per 10^7 cells. The cells were next incubated for 30 minutes on ice and then washed by centrifugation with a volume of buffer 10-fold that of the labeling volume and resuspended in 500 μL of buffer per 10^5 cells. CD11b+ cells were depleted using an MD depletion column (Miltenyi Biotec Inc.) placed in the magnetic field of a MidiMACS separation unit (Miltenyi Biotec Inc.).

Histochemical and immunohistochemical analysis of surgically resected samples

From the surgically resected lower gingival squamous cell carcinoma mandible samples, decalcified, H&E-stained specimens were prepared. Sections from the deepest part of the invasion and the boundary between the tumor and the bone were evaluated primarily by light microscopic observation. All the patients were examined and treated at Okayama University Hospital (Okayama, Japan) between 2000 and 2010, and the diagnosis was clinicopathologically confirmed. No patient had received chemotherapy and/or radiation therapy before surgery was done. All tumor samples were obtained with the consent of the patients.

The sections were sequentially dewaxed through a series of xylene, graded ethanol, and water immersion steps. After being autoclaved in 0.2% citrate buffer for 15 minutes, the sections were incubated with 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. A primary anti-mTOR (rabbit IgG), p-mTOR Ser2448 (rabbit IgG), Cell Signaling Technology) was used for the immunohistochemical analysis. The specimens were incubated with a 1:200 dilution of the primary antibody overnight at 4°C, followed by three washes with TBS. The slides were then treated with a streptavidin-biotin complex [Envision System labeled polymer, horse-radish peroxidase (HRP), Dako] for 60 minutes at a dilution of 1:100. The immunoreaction was visualized using a 3,3′-diaminobenzidine (DAB) substrate-chromogen solution (Dako Cytomation Liquid DAB Substrate Chromogen System, Dako), and counterstaining was done with hematoxylin. Finally, the sections were immersed in an ethanol and xylene bath and mounted for light microscopic observation.

Cell proliferation assay

The HSC-2 cells were plated in a 96-well plate at 1 × 10^4 cells per well in the presence of temsirolimus (Supplementary Fig. S1), which was obtained from Wyeth. A MTS assay was done to obtain a relative cell number after 72 hours of incubation under the experimental procedure (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Co.).

Immunoblot analysis

HSC-2, SAS, RAW264.7, or ST2 cells were rinsed once with ice-cold PBS and lysed in an ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.4), containing 150 mmol/L NaCl, 1% Triton X-100, 1% NP-40, 10 mmol/L NaF, 100 mmol/L leupeptin, 2 mg/mL aprotinin, and 1 mmol/L phenylmethyl sulfonyl fluoride]. Cell lysates containing 10 μg of total protein in a lysis buffer were electrophoresed in 12% SDS-PAGE gels and the proteins were transferred to nylon membranes (Immobilon-P, Millipore Co.). The membrane was incubated with primary and secondary antibodies according to the ECL chemiluminescence protocol (RPN2109, Amersham Biosciences) to detect secondary antibody binding. Antibodies against mTOR (rabbit IgG), p-mTOR Ser2448 (rabbit IgG), S6 (mouse IgG), p-S6 Ser235/236, Akt (rabbit IgG), p-Akt Ser473 (mouse IgG), IκBα (rabbit IgG), and p-IκBα Ser32/36 (mouse IgG) were purchased from Cell Signaling Technology and used at a 1:200 dilution. HRP-conjugated goat anti-rabbit antibodies or goat anti-mouse IgG were used as the secondary antibodies at a 1:1,000 dilution.

RNA extraction and reverse transcriptase-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies Inc.) according to the manufacturer’s instructions. cDNA was generated from 1 μg of total RNA using a first-strand cDNA synthesis kit (Invitrogen) in a final volume of 20 μL, then amplified for 30 cycles using two oligonucleotide primers: 5′-ACACCTCACCAT-CATGC-GC-3′ and 5′-GTACCCTTCCCGAGTATTT-3′ for receptor activator for nuclear factor-κB ligand (RANKL), 5′-ACA C A A A G T G A AT G C C G A G A G -3′, 5′-TCTGTGTTGAGTTGTCAGTCT-3′ for osteoprotegerin (OPG), and 5′-TGACGGGAAGCTCATTGG-3′ and 5′-TCCACCACCTGTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each PCR cycle was carried out for 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 68°C. The PCR products were then separated with 2% agarose gels containing ethidium bromide and visualized under UV light. The expected sizes of PCR products were 197 bp for RANKL, 191 bp for OPG, and 307 bp for GAPDH.

Tartrate-resistant acid phosphatase staining and osteoclast activity assay

RAW264.7 murine macrophage cells were treated with 50 ng/mL of recombinant mouse RANKL (PEPROTECH EC) for 5 days. The cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP; Sigma), and the number of TRAP-positive multinucleated cells (nuclear number >3) in each well was counted. For osteoclast activity assay, murine CD11b+ bone marrow cells were plated into a calcium phosphate apatite-coated substrate plate (Osteogenic Core Technologies) at a density of 1 × 10^6 cells/well. The cells were treated with RANKL (50 ng/mL) for 5 days and then incubated with both temsirolimus and RANKL (50 ng/mL) for >7 days. The remaining cells in the plate were lysed using 1 N NaOH with a 6% sodium hypochlorite solution. Five images per well were obtained using an inverted microscope (200%), and the resorbed area was measured using image software (Lumina Vision/OL).
Scratch assay

HSC-2 cells were grown to confluence on 6-well tissue culture dishes, and a single scratch was made in the confluent monolayer using a sterile 200-μL pipette tip. The monolayer was washed with PBS, and then complete medium containing 1, 10, or 50 nmol/L temsirolimus or vehicle alone was added. Serial photographs of the same scratched section were taken after 24 hours. The number of cells that had migrated over the margins of the wounds was counted after 24 hours of temsirolimus treatment.

Animal experiments

Human oral squamous cell carcinoma xenografts were established in 5-week-old female BALB/c nude mice (Clea Japan, Inc.) by s.c. inoculation of 8 × 10⁶ HSC-2 cells into the dorsal flank as described previously (13, 14). The mice were randomly assigned into two groups (n = 10/group). Each group of the mice was treated with i.p. injection of 200 μL solution containing temsirolimus (20 mg/kg; refs. 15, 16) or vehicle only twice a week (Clea Japan, Inc.) by s.c. inoculation of 8 × 10⁶ HSC-2 cells established in 5-week-old female BALB/c nude mice (Clea Japan, Inc.).

The expression of mTOR in osteolytic mandibular squamous cell carcinoma

Figure 1A illustrates a representative histologic pattern of invasive bone destruction observed in a patient with oral squamous cell carcinoma in the mandibular region. mTOR and phosphorylated mTOR (p-mTOR) were highly expressed in tumor cells that had invaded the bone matrix. Ten of ten cases of aggressive invasive phenotype showed a strong intensity of mTOR and p-mTOR immunoreactivity.

Inhibition of mTOR by temsirolimus suppresses cell growth and cell migration of HSC-2 oral squamous cell carcinoma cells

To analyze the antitumor effect of temsirolimus against oral squamous cell carcinoma in vitro, trypan blue exclusion and migration assays were done. As shown in Fig. 1B, temsirolimus significantly decreased the number of HSC-2 cells proportionately with an increasing dose in 72 hours after treatment. The IC₅₀ of a HSC-2 cell was 20 nmol/L (data not shown). The migration assay showed that the number of migrated HSC-2 cells was dose-dependently inhibited after temsirolimus treatment (P < 0.01; Fig. 1C). Subsequently, we analyzed the phosphorylation of mTOR and S6 after temsirolimus treatment in HSC-2 cells. Immunoblot analysis showed that the phosphorylation of mTOR Ser²⁴⁸⁸, S6 Ser²³⁵/²³⁶, and Akt...
Ser\textsuperscript{473} was decreased 15 minutes after temsirolimus treatment. However, temsirolimus did not change the expression of total mTOR, S6, Akt, or \( \beta \)-actin in both HSC-2 (Fig. 1D, left) and SAS (Fig. 1D, right) cells. These results suggest that temsirolimus decreases the phosphorylation of mTOR, S6, and Akt, and suppresses the cell viability and migration activity of oral squamous cell carcinoma cells.

Temsirolimus suppresses osteoclast formation and activity

Further, we studied the effect of temsirolimus on osteoclastogenesis. To elucidate the effect of temsirolimus in RANKL-mediated osteoclast formation, RAW264.7 murine macrophage cells, known as osteoclast precursor cells, were treated with RANKL (50 ng/mL) in the presence of temsirolimus for five days. Temsirolimus inhibited the number of TRAP-positive multinucleated osteoclast formation induced by RANKL in a dose-dependent fashion (Fig. 2A). To define the molecular mechanisms of the inhibitory effects of temsirolimus on osteoclast formation, we examined the effects of temsirolimus on signaling pathways induced by RANKL in RAW264.7 cells. Immunoblot analysis showed that mTOR and I\( \kappa \)B\( \alpha \) were transiently activated 5 minutes after RANKL treatment (50 ng/mL) in RAW264.7 cells (Fig. 2B). However, temsirolimus significantly blocked the RANKL-induced phosphorylation of mTOR and I\( \kappa \)B\( \alpha \) at a concentration of \( >10 \) nmol/L (Fig. 2C). Temsirolimus did not change the expression of total mTOR, Akt, and \( \beta \)-actin (Fig. 2B and C). Then, we analyzed the effect of temsirolimus on the bone resorptive activity of mature osteoclasts. Osteoclast activity assay using CD11b\textsuperscript{+} bone marrow cells showed that 50 and 100 nmol/L of temsirolimus significantly inhibited the bone resorptive activity of osteoclasts (\( P < 0.05 \) and 0.01, respectively; Fig. 2D). These results indicate that temsirolimus suppresses RANKL-induced osteoclast formation.
phosphorylation of mTOR and IκBα and inhibits osteoclast formation and function.

Temsirolimus suppresses oral squamous cell carcinoma xenografts in mice

To analyze the antitumor effect of temsirolimus in vivo, we established oral squamous cell carcinoma xenograft tumors derived from HSC-2 cells in nude mice. As shown in Fig. 3A and B, i.p. injection of temsirolimus significantly suppressed tumor growth compared with vehicle (P < 0.01). An important observation to note is that temsirolimus treatment did not cause body weight reduction, whereas significant body weight loss was seen in control mice (Fig. 3C). Temsirolimus did not affect body weight in non–tumor-bearing mice. There were no deaths during the experiments on mice.

Temsirolimus inhibits osteolytic bone destruction associated with invasion of oral squamous cell carcinoma in vivo

Smink et al. (18) suggested that mTOR signaling is important in the regulation of autophagy, a process

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**Figure 2.** The effects of temsirolimus on osteoclast formation and bone resorption. A, temsirolimus suppressed differentiation of RAW264.7 cells into osteoclast induced by RANKL. RAW264.7 cells were treated with RANKL (50 ng/mL) and the indicated concentration of temsirolimus in 96-well plates. Cells were fixed and stained for TRAP five days after treatment, and the number of TRAP-positive multinucleated cells (nuclear number >3) in each well was counted. B, detection of p-mTOR, mTOR, p-IκBα, and Akt expression by immunoblot analysis after RANKL (50 ng/mL) treatment in murine macrophage RAW264.7 cells. β-Actin served as the control. C, temsirolimus suppressed phosphorylation of mTOR and IκBα induced by RANKL in RAW264.7 cells. RAW264.7 cells were treated with RANKL (50 ng/mL) and the indicated concentration of temsirolimus for 5 minutes. Immunoblot analysis was done after the treatment. β-Actin served as the control. D, temsirolimus inhibited bone resorptive activity of mature osteoclasts. Bone resorptive activity of mature osteoclasts differentiated from CD11b+ bone marrow cells was evaluated in an osteoclast activity assay substrate as described in Materials and Methods. Similar results were obtained in at least three independent experiments. *, P < 0.05; **, P < 0.01, significant differences between the indicated groups.
Figure 3. The effect of temsirolimus on oral squamous cell carcinoma cells grown in nu/nu mice xenografts. A, macroscopic appearance of HSC-2 tumors in nu/nu mice 38 days after tumor inoculation. Top, tumors from control mice. Bottom, tumors from mice treated with temsirolimus. B, volume of tumor was monitored over time (days) after inoculation of tumor. Ten mice were used for each group. Tumor growth is expressed as mean tumor volume ± S.D. **, statistical significance defined as  P < 0.01. C, body weights of the tumor-bearing mice treated with temsirolimus or vehicle only, and non-tumor-bearing mice with temsirolimus as a control were monitored. All results are expressed as means ± S.D. *, statistical significance defined as  P < 0.05.

Proposed to be involved in osteoclast function, and we determined the effect of temsirolimus on osteolytic bone destruction induced by oral squamous carcinoma in vivo. As shown in Fig. 4A, the osteolytic lesions were clearly visible in the hind limbs of the mouse models of bone invasion by human oral squamous cell carcinoma treated with vehicle only. Surprisingly, few destructive lesions were detected in the hind limbs of the mouse group treated with temsirolimus. The total area of radiographic osteolytic lesions from all tibias was significantly suppressed by temsirolimus treatment compared with control (P < 0.05). Histologic analysis revealed that the tumor cells invaded the bone marrow space and destroyed both trabecular and cortical bone in control mice. In contrast, the bone remained intact after temsirolimus treatment. Histomorphometric analysis confirmed that the tumor area in the hind limbs after temsirolimus treatment was significantly suppressed compared with the control (Fig. 4B). In addition, the number of TRAP-positive osteoclasts was significantly less in the hind limbs of the mouse group treated with temsirolimus than in those of control mice (P < 0.05; Fig. 4C). These results suggest that temsirolimus significantly suppressed oral squamous cell carcinoma tumorigenicity, as well as osteoclastogenesis in osteolytic bone destruction associated with invasion of oral squamous cell carcinoma.

**Temsirolimus improves hypercalcemia and osteolysis induced by PTHrP**

To confirm the effects of temsirolimus on PTHrP-induced osteolysis in vivo, we used mouse models for humoral hypercalcemia of malignancy induced by PTHrP. As shown in Fig. 5A, blood calcium concentration increased and reached a maximum of 2.73 mmol/L for six days after PTHrP treatment. However, temsirolimus treatment improved the hypercalcemia induced by PTHrP, and calcium concentration remained at normal level (P < 0.05). Administration of vehicle did not affect the blood-free calcium concentration. We then measured the body weight of each mouse group after treatments. The body weight decreased and reached a minimum of 10.95 g for eight days after PTHrP treatment. However, temsirolimus significantly prevented the body weight loss by PTHrP injection (P < 0.05). The control vehicle–treated group showed no effect on mouse body weight (Fig. 5B). To analyze the effects of temsirolimus on hypercalcemia, we analyzed osteoclast formation in the coculturing system of ST2 cells as osteoblast lineage cells and CD11b+ bone marrow cells as osteoclast precursor cells in the presence of PTHrP with or without of temsirolimus. As shown in Fig. 5C, temsirolimus significantly inhibited TRAP-positive multinucleated osteoclast formation in a dose-dependent manner and completely suppressed osteoclast formation at a concentration of 50 nmol/L. Next, we examined RANKL and OPG mRNA expression after PTHrP treatment in bone marrow stromal ST2 cells. RT-PCR and immunoblot analysis showed that RANKL mRNA and protein expression mildly increased 24 hours after PTHrP treatment. Significantly, temsirolimus inhibited the upregulation of RANKL mRNA and protein expression after PTHrP treatment in a dose-dependent manner, and 100 nmol/L temsirolimus strongly reduced the RANKL mRNA and protein expression in ST2 cells. On the other hand, OPG mRNA and protein expression were reduced 24 hours after PTHrP treatment; however, temsirolimus reversed this effect at a dose of 1 nmol/L and significantly upregulated the OPG mRNA and protein expression at a dose of 100 nmol/L (Fig. 5D). These results suggest that downregulation of RANKL mRNA and protein expression and upregulation of OPG mRNA by temsirolimus in ST2 cells inhibited differentiation of CD11b+ bone marrow cells into mature osteoclasts in the coculturing system and the inhibition of osteoclastogenesis.
by temsirolimus treatment improved hypercalcemia after PTHrP treatment \textit{in vivo}.

\textbf{Discussion}

Previous studies have implicated a role for mTOR in oral squamous cell carcinoma and other malignancies. However, the role of mTOR involvement in bone destruction induced by oral squamous cell carcinoma is not well understood. In the present study, the inhibition of mTOR signaling with temsirolimus inhibited bone destruction associated with oral squamous cell carcinoma invasion not only by suppressing osteoclastogenesis and osteoclast function, but also by inhibiting tumor proliferation and migration. These results indicate that mTOR is a critical mediator of osteolytic bone destruction in the bone tumor microenvironment.

Here, we have shown that both HSC-2 cells and osteoclasts were potently and efficaciously inhibited by temsirolimus at the level of proliferation and osteoclast formation and activation (Figs. 1–3). Temsirolimus inhibited HSC-2 cell growth with an $IC_{50}$ of 20 nmol/L, whereas it inhibited RAW264.7 cell growth with an $IC_{50}$ of 54 nmol/L. The discrepancy in $IC_{50}$ could be due to differences in the cell systems, and HSC-2 cells might be more sensitive to temsirolimus than are RAW264.7 cells. HSC-2 cells might overexpress mTOR in the bone destructive region more significantly than in the primary site. Osteoclasts also might activate the mTOR signaling by RANKL signaling and binding to the bone matrix (19), and the osteolytic destruction site would be expected to be more sensitive than normal organs to mTOR inhibition.

To investigate the molecular mechanism of antiosteoclastic effects, we examined RANKL-induced signaling...
pathways in RAW264.7 murine macrophage cells after temsirolimus treatment. Our data showed that temsirolimus inhibited IκBα activation induced by RANKL in RAW264.7 murine macrophage cells (Fig. 2B and C). Our previous study showed that the inhibition of Akt suppressed phosphorylation of IκBα and NFκB DNA-binding activity, and blocked RANKL-induced osteoclastogenesis in mouse bone marrow macrophages (20).

RANKL is also reported to lead to the amplification of nuclear factor of activated T cells (NFATc1), known as the master transcription factor for osteoclast differentiation. These findings imply that the DNA binding of NFκB, IκBα, and NFATc1 has strongly taken part in the RANKL-induced osteoclastic effect. We also found that temsirolimus markedly inhibited and diminished the capacity to excavate apatite pits. Our data on osteoclast...
functional inhibition by temsirolimus is in agreement with a report that everolimus suppresses bone resorption and cathepsin K expression by osteoclasts (21).

It has been reported that various bone resorbing factors are produced and secreted from malignant tumor cells; in particular, PTHrP is involved in the pathogenesis of hypercalcemia associated with malignant tumors, including oral squamous cell carcinoma (22). In this study, temsirolimus improved hypercalcemia induced by PTHrP in vitro; this is the first report describing a role for mTOR in regulating hypercalcemia through the PTHrP signaling pathway in stromal cells. Our results are consistent with reports showing that everolimus suppresses the OVX-induced loss of cancellous bone (21), the lack of effect of rapamycin on bone (23), and other immunosuppressive drugs, such as corticosteroid and cyclosporine, exerting negative bone effects (24, 25). To better understand the mechanisms of mTOR signaling on PTHrP-induced hypercalcemia, we used a coculturing system of bone marrow stromal ST2 cells and murine CD11b+ bone marrow cells as osteoclast precursor cells in the presence of PTHrP with or without of temsirolimus. Stromal cells are essential for osteoclastogenesis through a cell-to-cell interaction with osteoclast progenitor cells (26). Temsirolimus increased OPG mRNA expression and reduced RANKL mRNA expression in murine bone stromal ST2 cells after PTHrP treatment and resulted in suppressing differentiation of murine CD11b+ bone marrow cells into osteoclasts in the coculturing system. These results suggest that temsirolimus inhibits osteoclast formation by suppressing osteoblast function.

In summary, this study is, to the best of our knowledge, the first one to show that mTOR should be considered a target for the treatment of bone osteolysis induced by oral squamous cell carcinoma, and our findings strongly suggest that single use of temsirolimus or combination treatment of temsirolimus with other agents might be considered as an attractive approach to treat advanced oral squamous cell carcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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