Effect of YM529 on a Model of Mandibular Invasion by Oral Squamous Cell Carcinoma in Mice

Nianhui Cui, Takeshi Nomura, Hiroyasu Noma, Keiko Yokoo, Ryo Takagi, Sadamitsu Hashimoto, Masato Okamoto, Mitsunobu Sato, Guangyan Yu, Chuanbin Guo, and Takahiko Shibahara

Abstract

Purpose: This study examined the mechanisms of osteoclast-mediated bone invasion in a model of oral squamous cell carcinoma (OSCC). C3H/HeN mice were inoculated with SCC VII cells into the masseter region to establish an animal model of mandibular invasion by OSCC.

Experimental Design: The mice were divided into three groups: a control group, given daily s.c. injections of saline; group 1, given 2 μg per mouse per day of the bisphosphonate YM529; and group 2, given 10 μg per mouse per day of YM529. After 3 weeks of treatment, the lesions were studied by micro-computed tomography. After tartrate-resistant acid phosphatase (TRAP) staining, the osteoclasts were easily identified, and the percentages of the area occupied by osteoclasts were calculated by computer for each sample. The tumors were analyzed by RT-PCR to determine the mRNA expression of interleukin-6 (IL-6), parathyroid hormone–related protein (PTHrP), tumor necrosis factor-α (TNF-α), receptor activator of nuclear factor-κB (RANK), RANK ligand (RANKL), and osteoprotegerin.

Results: SCC VII cells rapidly multiplied in the masseter muscle of the mice. Bone invasion was evident only in the control group on micro-computed tomography. On TRAP-stained slices, the percentages of osteoclasts in groups 1 and 2 were significantly lower than that in the control group. The mRNA expressions of IL-6, PTHrP, THF-α, and RANK decreased as the concentration of YM529 increased.

Conclusions: We conclude that various cancer-derived cytokines play important roles in the invasion of bone by OSCC. YM529, a third-generation bisphosphonate, can suppress osteoclast-mediated bone invasion by OSCC. The mechanism of this effect might involve inhibition of cytokines such as IL-6, PTHrP, TNF-α, and RANK by YM529.

Oral squamous cell carcinoma (OSCC) is a common malignant tumor in the oral and maxillofacial region. Mandibular invasion by tumor requires surgical resection of the involved bone, potentially compromising mandibular function. How OSCC invades bone and how to prevent such invasion remain unsolved problems (1, 2).

Osteoclasts are more abundant in bone invaded by cancer than in normal regions of bone (1). The cytokines, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), are known to increase bone resorption by stimulating both osteoclast activation and differentiation. Levels of IL-6, parathyroid hormone–related protein (PTHrP), and TNF-α are significantly higher in cases of human gingival squamous cell carcinoma with bone invasion than in cases without such invasion (3). Synergistic activity of these substances produced by squamous cell carcinomas (SCC) are responsible for a marked increase in osteoclastic bone resorption and for malignancy-associated hypercalcemia (4). Recent observations have shown that members of the TNF family of ligands and receptors are critical regulators of osteoclastogenesis. Osteoprotegerin, a decoy receptor, was initially identified. Its ligand, receptor activator of nuclear factor-κB ligand (RANKL), was quickly found and shown to be expressed on stromal cells and osteoblasts. The cognate receptor of RANKL, RANK, is expressed in high levels on osteoclast precursors. Interactions between RANKL and RANK were shown to be required for osteoclast formation (5, 6). The combination of RANK and RANKL induces differentiation from pre-osteoclasts to osteoclasts, promoting bone invasion. Osteoprotegerin can also bind to RANKL by competing with RANK, which could protect against bone invasion (7, 8).

Bisphosphonates are analogues of endogenous pyrophosphate. They are potent inhibitors of osteoclastic bone resorption. For more than 20 years, bisphosphonates have been used to treat cancer-induced hypercalcemia, Paget’s disease, and osteoporosis (2, 9). Whether bisphosphonates prevent invasion...
of bone by metastasis has been experimentally studied in models of breast cancer and lung cancer (10). Although a third generation of bisphosphonates has already been developed and animal models of metastasis to the oral and maxillofacial region have been established (11), previous studies have only experimentally investigated the effect of bisphosphonates on bone invasion in a breast cancer model but not in oral cancer (10, 12).

To gain insight into the mechanism and prevention of bone resorption caused by OSCC, we established a model of maxillary bone invasion associated with OSCC and evaluated the effect of YM529 (Yamanouchi Pharmaceutical Co., Ltd., lot no. K5290019), a newly developed third-generation bisphosphonate, on such invasion. We also examined the expression of six cytokines expressed after treatment with this bisphosphonate.

**Materials and Methods**

**Cell culture.** SCC VII cells, derived from a cell line of mouse OSCC, were cultured in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (JRH Bioscience ASC Co., Lenexa, KS) and 1% antibiotic-antimycotic (Invitrogen Co., Auckland, NZ) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. When the cells reached confluency in 100 mm dishes (Falcon, Lincoln Park, NJ), the concentration of the cell concentration was approximately 1.3 \times 10^5/mL. The cells were collected by centrifugation, and the cell concentration was adjusted to 1.0 \times 10^5/mL with DMEM.

**Animal model.** Sixty-three male C3H/HeN mice weighing about 20 g at 8 to 10 weeks old each were used to establish a model of mandible invasion by OSCC. They were obtained from CLEA (Tokyo, Japan) and maintained under specific pathogen-free conditions throughout the experiment. Animal experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. The mice were randomly divided into three groups with similar average body weights (19.47, 19.90, and 20.11 g, respectively). They were anesthetized with ether, and 0.2 mL of SCC VII (1.0 \times 10^5/mL) in DMEM was injected s.c. into the left masseter region. In our mandible invasion models, tumor usually enlarge in about 3 to 4 weeks and subjects died after that.

**Experimental protocol.** Figure 1 shows the chemical structure of YM529. In the control group, 0.2 mL of saline was injected s.c. daily. The two other groups were given a daily s.c. injection of a solution (0.2 mL) of YM529 in a dose of either 2 \mu g per mouse per day (group 1) or 10 \mu g per mouse per day (group 2). The doses of YM529 have been described previously (13).

Treatment was given for 3 weeks. On days 1, 7, and 14, the mice were weighed and the longest diameters of the mass in the buccal region were measured in three directions. At the end of week 3, all surviving mice were sacrificed. The tumors were resected and preserved at \(-70°C,\) and the heads of the mice were fixed in 10% formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Micro-computed tomography and three-dimensional reconstruction.** Mice with the three largest tumors were identified in each group. Micro-computed tomography (CT) scans of the heads of these mice were taken and reconstructed with a KMS-755 Microtomographic System (Pony Co., Kashimura, Osaka, Japan) and a KMS-755 Microfocus CT Rebuilder (Version 1.00, Image Script Co., Ltd., Kashimura, Osaka, Japan). The photographs were reconstructed with a software package (TRI/3D Bon; R 2.1.16-S Ratoc, Tokyo, Japan).

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**Fig. 1.** Chemical structure of YM529: minodoronic acid hydrate, \([1\text{-hydroxy-2-}\text{(imidazo[1,2-}a\text{]pyridine-3-yl)ethylidene}]\text{bisphosphonic acid monohydrate.}\)
The tartrate-resistant acid phosphatase staining and counting of osteoclasts. Four-micron-thick frontal sections were made through the center of the tumor. The slices were stained serially with a tartrate-resistant acid phosphatase (TRAP) Kit (Hokudo Co., Sapporo, Japan) and Mayer’s hematoxylin (Mutoo Pure Chemicals Ltd., Tokyo, Japan). TRAP stain, a marker enzyme for osteoclasts, was done by incubating for 60 minutes at 37°C with a TRAP kit. Histomorphometrical determination of the area of osteoclasts of the tumor/bone interface was assessed at sites of invasion in mandibular specimens that had been stained with TRAP. The bone invasive tumor were identified using digital photographs that were taken with Axioplan2 and Axiophot2 (Carl Zeiss Co., Ltd., Jena, Germany). The photographs were analyzed with a software package (Image-Pro Plus, the Proven Solution Vision 4.5.1.23; Media Cybernetics, Inc., San Diego, CA) to calculate the area of osteoclasts for each slice. The calculation was done in four steps:

1. Photographs of regions where carcinoma was in contact with the bone were taken at a magnification of 200× microscope and saved with the addition of a 100 μm scale (Fig. 2A).
2. A 100 μm region of the tumor at the borderline of the carcinoma and bone was divided into four layers, each 25 μm in height (Fig. 2B).
3. The osteoclasts in each layer were recognized on the basis of TRAP staining and the software package (Fig. 2C).
4. Both the areas of osteoclasts and those of the layers were measured. The percentages of osteoclasts in each layer were then calculated (Fig. 2D).

Preparation of mRNA and semiquantitative RT-PCR. Part of the tumor was resected, and tumor cells were analyzed to investigate the mRNA expressions of IL-6, PTHrP, TNF-α, RANK, RANKL, and osteoprotegerin by RT-PCR. After resection from the head, the tumors were frozen at −0°C. Total RNA was isolated from the tumor with the use of TRizol reagent in accordance with the instructions of the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). The concentrations of total RNA were adjusted to 1 μg/32 μL. Then, 5 μg RNA samples were converted into cDNA by using a Ready-To-Go kit, following the instructions of the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). One micro-liter of cDNA was used for the amplification reaction. For each primer sequence, amplification was done for 35 cycles, with an initial denaturation step at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and an additional 7-minute extension at 72°C (PCR System 9700, Perkin-Elmer, Norwalk, CT). The primer sequences of IL-6, PTHrP, TNF-α, RANK, RANKL, and osteoprotegerin are shown in Table 1. Amplification products were analyzed in 1.5% agarose gel, stained with ethidium bromide, and photographed.

Statistical analysis. To assess the statistical significance of body weights, tumor sizes, and the percentage of the area that osteoclasts occupied in each layer, Student’s t test was done. Differences of means among the percentage of mRNA of six cytokines in three groups were compared by χ² test. A value of P < 0.05 was considered statistically significant (SPSS 11.0 for Windows 2001 software).

Results

Body weights and tumor sizes of animals treated with YM529. During the 3 weeks of treatment, 44 of 63 mice survived and 19 died of SCC. Body weight did not change in all groups during

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Table 1. Primer sequences

<table>
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<tr>
<th>Type</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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</thead>
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<tr>
<td>RANK</td>
<td>GACAGACAAAATGCAAACCTTGT</td>
<td>GTCTCTGGAAACCACATCTTCTCC</td>
</tr>
<tr>
<td>RANKL</td>
<td>TTTGCAAGGACTGACACTGAGG</td>
<td>TCCCTCCTCTCATGATGCTAGG</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>TGAGAGAGGATAAACCGG</td>
<td>CTTAGTTATAAGCAGCTTAT</td>
</tr>
<tr>
<td>IL-6</td>
<td>GTCTATACCCATCACAGATCCTTC</td>
<td>TGGAGTCGTCGTTAGCCTAGCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ACCCTCAACACTGACATGCAGTCCTTC</td>
<td>CAGATGAGACTAGCGAGCTGAGTT</td>
</tr>
<tr>
<td>PTHrP</td>
<td>AGAGTCTAACCAGGACAGGCC</td>
<td>GCGAGACATTTCTACTTCCCC</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>CCATGGAGAAAGCTGGG</td>
<td>CAAAGTTGTCATGGATGACC</td>
</tr>
</tbody>
</table>

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Fig. 3. Body weights (A) and tumor sizes (B) of animals treated with YM529. Bars, SD; *, no significance (P > 0.05); **, P < 0.05; ***, P < 0.01 by Student’s t test versus control group.
the three weeks. The differences in tumor weight were not significant among the three groups. The tumors transplanted into mice in the control group grew rapidly. However, in groups 1 and 2, which were treated with YM529, tumor growth was depressed. After 3 weeks, mean tumor diameter was 2,263.1 mm$^3$ in the control group, 1,019.7 mm$^3$ in group 1, and 459.2 mm$^3$ in group 2 (Fig. 3).

Three-dimensional image analysis using micro-CT. Micro-CT showed bone invasion only in the control group, but not in either group given YM529. In the control group, the zygoma and mandible were severely destroyed. The angle of the mandible had nearly disappeared (Fig. 4A and B). In groups 1 and 2, the mandible and zygoma were deformed by tumor enlargement; the mandibles were obviously asymmetric and the zygomas were bowed (Fig. 4C and D).

Appearance of resorption in the mandibular bone and an area of osteoclast occupation in the tumor/bone interface. After TRAP staining, osteoclasts were easily identified. Two types of osteolytic bone resorption were seen:

1. Direct invasion: osteoclasts were found at the border between tumor and bone. The bone was invaded in a tooth-like manner. These findings were common in the control group (Fig. 5A).
2. Normal modification: osteoclasts, which modified normal bone, were found around normal bone (Fig. 5B).

The percentage of areas occupied by osteoclasts is shown in Table 2. The percentage of osteoclasts decreased as the distance from the tumor border increased. Not only the total area occupied by osteoclasts in each slice but also the areas occupied by osteoclasts in each of the four layers differed significantly between the control group and the two groups treated with YM529.

mRNA expression of the osteoclast-related cytokines. Expressions of mRNA of the six cytokines examined (IL-6, TNF-α, PTHrP, RANK, RANKL, and osteoprotegerin) were found in all three groups. The expression of IL-6, TNF-α, PTHrP, and RANK mRNA were significantly more decreased in groups 1 and 2 than in the control group. Osteoprotegerin mRNA expression...
increased and RANKL decreased with an increase in the concentration of YM529, but this trend was not significant. From these results, it became clear that the production of IL-6, TNF-α, PTHrP, and RANK mRNA was inhibited by administration of YM529 (Table 3).

**Discussion**

Animal models are essential for studies of the mechanism of bone invasion by OSCC. Takahashi et al. (14) transplanted carcinoma cells into tooth extraction sockets to create a model of bone invasion by tumor. Cancer cells have also been directly injected into the mouth floor (15). SCC VII is a malignant tumor cell line derived from the mouse that grows without being affected by the immune system. This cell line was first used to develop an animal model of oral cancer of the mouth floor. Tumor growth, cervical lymph node metastasis, and pulmonary metastases have been confirmed (15, 16). In the present study, SCC VII cells were injected into the masseter region of mice. Both tumor growth and bone invasion were confirmed. Micro-CT revealed severe destruction of the mandible and zygoma in the control group. This finding was confirmed histologically. After TRAP staining, high densities of osteoclasts were found around bone invaded by tumor. These characteristics indicated that a mouse model of bone invasion by SCC had been successfully established.

Early experiments showed that bisphosphonates inhibit dissolution of calcium phosphate crystals *in vitro*. In *vivo*, bisphosphonates inhibit bone resorption both in normal animals as well as in animals in which bone resorption is stimulated experimentally (17). These findings led to bisphosphonates being used for the treatment of diseases such as osteoporosis and Paget's disease (18). Bisphosphonates were subsequently found to be useful for the treatment of malignant osteolysis. Experimental evidence has suggested that bisphosphonates act directly on tumor cells, either by inhibiting mechanisms responsible for bone metastasis or by inducing apoptosis of tumor cells (19). Thus, YM529, the molecular mechanism of which remains unknown, probably has anti-tumor activity. YM529 is a third-generation bisphosphonate that prevents bone invasion caused by metastases from breast cancer and lung cancer (10, 12). In our study, YM529 inhibited tumor invasion of the mandible and zygoma. In both groups 1 and 2, deformity of the mandible and zygoma caused by tumor enlargement was confirmed on micro-CT. The mandibles in these groups were obviously asymmetric and the zygomas were bowed.

**Table 2.** The percentage of the area that osteoclasts occupied in each layer

<table>
<thead>
<tr>
<th>Layer</th>
<th>Layer 1</th>
<th>Layer 2</th>
<th>Layer 3</th>
<th>Layer 4</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>Mean (SD)</td>
<td>11.70 (7.59)</td>
<td>8.40 (7.69)</td>
<td>8.23 (17.27)</td>
<td>4.75 (5.94)</td>
</tr>
<tr>
<td>Group 1</td>
<td>Mean (SD)</td>
<td>6.92* (5.88)</td>
<td>2.10* (3.07)</td>
<td>1.10 (1.90)</td>
<td>0.61* (1.02)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Mean (SD)</td>
<td>5.67* (4.75)</td>
<td>1.01* (1.25)</td>
<td>0.43* (0.57)</td>
<td>0.32* (0.53)</td>
</tr>
</tbody>
</table>

*P < 0.01 versus control group by two simple t tests.

†P < 0.05 versus control group by two simple t tests.

Fig. 5. Two types of osteolytic bone resorption. Osteoclasts (violet) were at the borderline between tumor and bone (A). The bone was invaded by the carcinoma. Osteoclasts (violet) were at the surface of normal bone (B). The osteoclasts took part in the normal modification of the bone.
Table 3. Percentages of mRNA of six cytokines in three groups

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>TNF-α</th>
<th>PTHrP</th>
<th>RANK</th>
<th>RANKL</th>
<th>Osteoprotegerin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>56.25</td>
<td>68.75</td>
<td>93.75</td>
<td>75.00</td>
<td>68.75</td>
<td>50.00</td>
</tr>
<tr>
<td>Group 1</td>
<td>8.33*</td>
<td>91.67</td>
<td>41.67*</td>
<td>67.67</td>
<td>67.00</td>
<td>50.00</td>
</tr>
<tr>
<td>Group 2</td>
<td>0</td>
<td>12.50*</td>
<td>18.75*</td>
<td>37.50*</td>
<td>56.25</td>
<td>56.25</td>
</tr>
</tbody>
</table>

* P < 0.01 versus control group by χ² test of cross-tabulation.
† P < 0.05 versus control group by χ² test of cross-tabulation.

TRAP is an accepted marker for the identification of osteoclasts (20, 21). It can also identify pre-osteoclasts (22). Cytomorphometric analysis can be done after TRAP staining for osteoclastic populations with the use of an automatic image analyzer and a computerized image analysis system (23, 24).

We used this technique to count osteoclasts. As compared with the control group, fewer osteoclasts were seen after TRAP staining in the treated groups. In some samples, osteoclasts were found beneath the periosteum, and the tumor did not penetrate the periosteum. These findings indicated that bone resorption was caused by tumor-induced pressure rather than by direct invasion and may account for the deformity of the mandible. Inhibition of bone resorption on TRAP staining was also related to the concentration of YM529. The rate and level of bone resorption could be estimated on the basis of the percentages of osteoclasts. In our study, the percentages of osteoclasts differed significantly between the control group and the two YM529 groups. Differences in osteoclast densities might underlie the differences in bone resorption on micro-CT. Our findings suggest that YM529 can inhibit SCC-induced bone resorption by reducing the number of osteoclasts. Besides suppressing differentiation from pre-osteoclasts to osteoclasts, bisphosphonates could also inhibit tumor growth or have antitumor potential (25). Some studies have shown that bisphosphonates cause apoptosis of osteoclasts (26–28), consistent with the results of our study. Tumor sizes in the control group were larger than those in the two YM529 groups. Although these differences were significant, the mechanism responsible for the difference in tumor size remains to be investigated.

Many cytokines participate in bone metabolism by acting on osteoclasts and osteoblasts (2, 7, 29, 30). As mentioned above, we studied six cytokines (IL-6, PTHrP, TNF-α, RANK, RANKL, and osteoprotegerin). The mRNA expression levels of IL-6, PTHrP, and TNF-α were much higher in the control group than the two groups treated with YM529. These three cytokines can strongly induce osteoclasts to invade bone. Therefore, they might be derived from tumor cells as well as being inhibited by YM529. Bisphosphonates are pyrophosphate analogues that include potent inhibitors of bone resorption. These compounds act directly on osteoclasts, suppressing isoenzymation by inhibiting sennyl diphosphate synthase in the cholesterol pathway, causing osteoclast inactivation. Bisphosphonates thereby reduce bone loss caused by parathyroid hormone and PTHrP (31). The RANK-RANKL-osteoprotegerin system regulates the differentiation of osteoclasts. Binding between RANK and RANKL may induce differentiation from pre-osteoclasts to osteoclasts and activate osteoclasts. Osteoprotegerin blocks binding of RANK and RANKL by competing with RANK (2, 7).

In our study, the three groups showed different levels of mRNA expression. The level of RANK mRNA expression was obviously higher in the control group, suggesting that YM529 inhibited the expression of RANK in a concentration-dependent manner. Osteoprotegerin mRNA expression increased and RANKL mRNA expression decreased with an increase in the concentration of YM529, although there were no significant differences among the three groups. These trends might have been significant if more samples had been studied. Changes in cytokine expression may have strongly inhibited binding between RANK and RANKL, decreasing the number of osteoclasts. Thus, YM529-induced inhibition of the expression of osteoclast-related cytokines might at least partially account for the osteoclast-mediated suppression of bone invasion in OSCC.

In conclusion, our results strongly suggest that various cancer-derived cytokines play an important role in bone invasion by OSCC. YM529, a third-generation bisphosphonate, can suppress osteoclast-mediated bone invasion by OSCC. The mechanism of this effect might involve inhibition of cytokines such as IL-6, PTHrP, TNF-α, and RANK by YM529.

References


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