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Biochemical and Biophysical Research Communications 328 (2005) 91–97
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Nitrogen-containing bisphosphonate, YM529/ONO-5920 (a novel minodronic acid), inhibits RANKL expression in a cultured bone marrow stromal cell line ST2

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Received 4 December 2004
Available online 5 January 2005

Abstract

Increase in bone resorption by osteoclasts can cause metabolic bone diseases, such as osteoporosis. Recent attention has been paid to the receptor activator of the NF-κB ligand (RANKL), an accelerator of osteoclast differentiation. RANKL is expressed on the bone marrow-derived stromal cell membrane and induces the differentiation of osteoclasts by binding to RANK expressed on the osteoclast precursor cell membrane. Since the inhibition of RANKL expression can lead to the inhibition of osteoclastic bone resorption, the clinical application of RANKL inhibition could be expected to have a major effect on metabolic bone disease therapy. In this study, we investigated whether or not YM529/ONO-5920, a nitrogen-containing bisphosphonate (a novel minodronic acid), inhibits RANKL expression in a bone marrow-derived stromal cell line (ST2 cells). Reverse transcription-polymerase chain reaction revealed that the administration of YM529/ONO-5920 to ST2 cells inhibited RANKL mRNA expression and reduced RANKL proteins as assessed by Western blot analysis. The inhibition of RANKL mRNA expression was reversed when geranylgeranyl pyrophosphate (GGPP), an intermediate in the mevalonate pathway, was used in combination. Furthermore, YM529/ONO-5920 reduced phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), and similarly, U0126, a mitogen-activated protein kinase kinase 1/2 inhibitor, inhibited RANKL expression. Pretreatment with GGPP reversed the YM529/ONO-5920-induced decrease in phosphorylation of ERK. Furthermore, YM529/ONO-5920 decreased TRAP-positive cells in co-culture of ST2 cells and an osteoclast cell line, C7 cells, and this decrease was inhibited by pretreatment with GGPP. This indicates that YM529/ONO-5920 inhibits GGPP biosynthesis in the mevalonate pathway and then signal transduction in the Ras–mitogen-activated protein kinase pathway, thereby inhibiting RANKL expression on ST2 cells. These results suggest a newly elucidated action of bisphosphonates in the inhibition of bone resorption.

Keywords: Bisphosphonate; YM529/ONO-5920; RANKL; ST2 cells; Stromal cells

Bone tissue is continuously remodeled through bone resorption by osteoclasts and bone formation by osteoblasts, thus maintaining a dynamic balance in bone quantity. Metabolic bone diseases, such as osteoporosis, are considered to be conditions in which this bone resorption–formation balance is lost in favor of increased bone resorption by osteoclasts [1].

Osteoclasts are large multinucleated cells derived from hemopoietic cells, such as granulocyte/macrophage-colony stimulating factor (GM-CFU). It has been considered that the differentiation of osteoclasts from

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doi:10.1016/j.bbrc.2004.12.145

their precursor cells requires interaction between osteoclast precursor cells and osteoblasts [2]. However, recent reports have suggested that the interaction of osteoclast precursor cells with bone marrow-derived stromal cells is also of key importance, and this interaction is attracting attention [2–8]. Factors deeply involved in these cellular interactions include macrophage-colony stimulating factor (M-CSF) and RANKL, which are expressed on osteoblasts and stromal cells. RANKL in particular is considered essential to the differentiation of osteoclasts [3–8]. It is clear that osteoclast differentiation is induced by RANKL bound to RANK expressed on the osteoclast precursor cell membrane. Moreover, it has been reported that RANKL expression is increased by bone resorption accelerators, such as 1,25-$(OH)_2$ vitamin D_3 ($V.D_3$), IL-1,6, prostaglandin E_2, parathyroid hormone, and tumor necrosis factor (TNF)-α [1,7,8]. Since the inhibition of RANKL expression can lead to the inhibition of bone resorption by osteoclasts, its clinical application would have a major influence on osteoporosis therapy. Although nitrogen oxide (NO) [9] and TGF-β1 [10] have been reported to inhibit RANKL expression in the bone marrow-derived stromal cell line of ST2 cells, their clinical application remains to be achieved.

Bisphosphonates used for osteoporosis therapy are structurally analogous to pyrophosphate found in vivo. As a consequence of their great affinity to bone minerals, bisphosphonates are considered to rapidly accumulate in bone tissue and induce apoptosis when taken up by osteoclasts during bone resorption [11]. It has also been reported that nitrogen-containing bisphosphonates induce apoptosis by inhibiting farnesyl pyrophosphate (FPP) and GGPP biosynthesis in the cholesterol biosynthetic pathway in osteoclasts, and thus inhibit prenylation of low-molecular G-proteins important in signal transduction, such as Ras, Rho, and Rab [12,13]. A newly developed bisphosphonate, YM529/ONO-5920, in particular has a marked inhibitory action on GGPP biosynthesis, and the action is also observed in hemopoietic tumor cells [14], which, unlike osteoclasts, do not cause bone resorption.

These findings raise the possibility that YM529/ONO-5920, a newly developed bisphosphonate, affects not only osteoclasts but also stromal cells, and inhibits osteoclast formation by inhibiting RANKL expression. To test this hypothesis, we investigated whether or not YM529/ONO-5920 inhibited RANKL expression in the bone marrow-derived stromal cell line of ST2 cells and defined its mechanism of action.

Materials and methods

Materials. YM529/ONO-5920 (1-hydroxy-2-(imidazo-[1,2a]pyridin-3-yl) ethylidene bisphosphonic acid monohydrate) was supplied from Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). Dexamethasone (DEX) was purchased form Wako. These regents were dissolved in phosphate-buffered saline (0.05 M, pH 7.4), filtered through Syringe Filters (0.45 μm, IWAKI GLASS, Japan), and used for various assays described below.

GGPP and $V.D_3$ were purchased from Sigma. GGPP was dissolved in dry ethanol. U0126 were purchased from Promega and dissolved in DMSO. And then, these dissolved regents were resuspended in phosphate-buffered saline (0.05 M, pH 7.4) filtered through Syringe Filters (0.45 μm, IWAKI GLASS) before use.

Cell culture. We used ST2 cells, which are mesenchymal stem cells that have the ability to differentiate into osteoblast-like cells and which greatly express RANKL in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX. The ST2 cells were provided by Riken Cell Bank and cultured in RPMI1640 medium (Nissui) supplemented with 10% fetal calf serum (Gibco), penicillin (100 μg/ml, Meiji), streptomycin (100 U/ml, Meiji), and 25 mM Hepes (pH 7.4, Wako) in an atmosphere containing 5% CO_2.

Co-culture with C7 cells. ST2 cell suspension adjusted to 3×10^4 cells/ml was distributed to a 24-well plate at 1 ml/well. After preculture for 24 h, YM259/ONO-5920 was added so as to adjust the final concentration to 0.5 and 1 μM.

After 24 h, 10^{-8} M $V.D_3$ (Sigma) and 10^{-7} M dexamethasone (DEX, WAKO) were added, and the cells were cultured for 3 days, followed by seeding of C7 cell [15] suspension adjusted to 5×10^3 cells/ml. The half-volume of culture medium was exchanged with fresh medium every 3 days, and the final concentration of YM529/ONO-5920 was adjusted to 0.5 and 1 μM. After culturing for 12 days, TRAP staining was performed, and TRAP-positive cells with 3 or more nuclei were counted under a light microscope (100×).

After culturing ST2 cells, the cells were co-cultured with C7 cells, having the potential to differentiate into osteoclasts, and TRAP-positive multinucleated cells (3 or more nuclei) were counted under a microscope after 12 days.

Cell viability. Cell viability was assessed by the tetrazolium dye method using TetraColor ONE assay kit (Seikagaku). Cells (5×10^3 cells/well) were plated in 96-well plates and incubated with 0.5, 1, 5, and 10 μM YM529/ONO-5920 for 3 and 6 days. Absorbance was measured at 492 nm using a microplate reader (SK601, Seikagaku).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cultured ST2 cells with the use of Trizol (Invitrogen, USA). The pellet of total RNA was washed briefly with 75% ethanol and resuspended in 30 μl DEPC-treated water. The concentration of the total RNA was determined by measuring the optical density at 260 nm with the use of a spectrophotometer.

Single-strand cDNA was synthesized from 1.0 μg of total RNA by incubating with oligo$(dT)_{12–18}$ primer and SuperScriptTMII RNase H-reverse transcriptase using SuperScript Fist-Strand Synthesis System for RT-PCR (Invitrogen, USA).

cDNA was used as a template for polymerase chain reaction amplification to generate products corresponding to mRNA encoding the various gene products. Each PCR contained cDNA, dNTPmix (Takara Biomedical), 10× PCR buffer (Takara Biomedical), and Pyrobest (Takara Biomedical). DNA was amplified under the following typical cycling conditions: denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min, extension at 72 °C for 0.5 min (30 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), and denaturation at 94 °C for 0.5 min, annealing at 61 °C for 0.5 min, and extension at 72 °C for 0.5 min (30 cycles for RANKL). PCR was performed in a DNA thermal cycler (Takara PCR thermal cycler MP, Takara Biomedical). The following primers used were: RANKL, 5′-GGT CGG GCA ATT CTG AAT T-′3 (5′-primer); 5′-GGG GAA TTA CAA AGT GCA CCA G-3′ (3′-primer) and GAPDH, 5′-ACT TTG TCA AGC TCA TTT-3′ (5′-primer), 5′-TGC AGC GAA CTT TAT TG-3′ (3′-primer). Then, the PCR product was mixed with bromophenol blue-loaded buffer and electrophoretically separated in a 2% agarose gel in TAE buffer. After staining with ethidium bromide,

bands of the PCR products were visualized with ultraviolet light and recorded with Coolsaver (ATTO).

Western blotting. ST2 cells treated with various conditions were lysed with lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 mM PMSF). The protein content of this cell lysate was determined using the BCA protein assay kit (Pierce). Each extract (40 μg protein) was fractionated on a polyacrylamide–SDS gel and then transferred to PVDF membrane (Amersham). The membrane was incubated for 30 min for blocking in TBS buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl) containing 3% skim milk. After having been washed three times with 0.1% Tween 20 in TBS, the membrane was incubated for 1 hour at room temperature with each antibody (anti-phospho ERK1/2 antibody, anti-ERK1/2 antibody (Cell Signaling Technology), and anti-RANKL antibody (Santa Cruz)) in TBS buffer. After three more washes with the TBS–Tween buffer, the membrane was incubated for 1 h at room temperature with anti-rabbit IgG sheep antibody linked to HRP (Amersham). Immunoreactive proteins were visualized by the ECL-plus detection system (Amersham). As the internal standard, anti-β-actin mouse monoclonal antibody (Sigma) was used as the primary antibody to detect β-actin protein.

Statistical analysis. Statistical analysis was performed with the Mann–Whitney test and a value of $P < 0.01$ was considered statistically significant.

Results

Determination of the YM529/ONO-5920 concentrations administered to ST2 cells

Since high concentrations of YM529/ONO-5920 induce cell death in ST2 cells, we determined a concentration that would not induce their death.

We determined the cell survival rate, which was defined as the number of living cells at 3 and 6 days after receiving YM529/ONO-5920 at concentrations of 0.5, 1, 5, and 10 μM as compared with the number of live control (untreated) cells. The survival rates at the respective concentrations were 99.8%, 100.2%, 100.6%, and 97.9% on Day 3 and 97.8%, 97.0%, 93.7%, and 82.8% on Day 6, respectively (Fig. 1). Based on these results, we selected 5 μM as the concentration of YM529/ONO-5920 that would not induce cell death of ST2 cells.

Inhibition of RANKL mRNA and protein expression in ST2 cells receiving YM529/ONO-5920

We investigated the effects of YM529/ONO-5920 on RALKL mRNA and protein in ST2 cells receiving YM529/ONO-5920 in the presence of $V.D_3$ and DEX.

RANKL mRNA expression was observed in untreated ST2 cells. In the $V.D_3$ and DEX group, RANKL mRNA expression was increased (Fig. 2A). The administration of YM529/ONO-5920 markedly inhibited RANKL mRNA expression (Fig. 2A).

We then investigated whether RANKL protein expression was actually inhibited in ST2 cells receiving $V.D_3$ and DEX or YM529/ONO-5920.

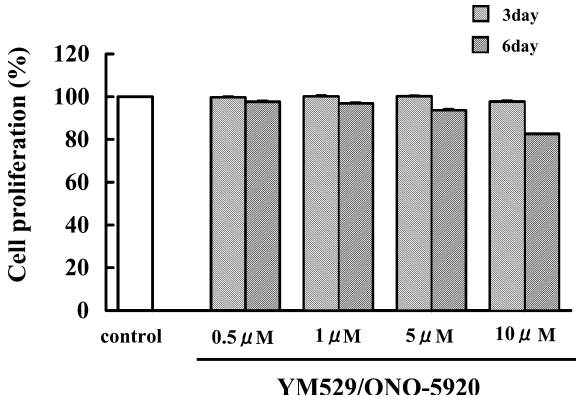

Fig. 1. Determination of the YM529/ONO-5920 concentrations administered to ST2 cells. Cells (5000 cells/well) were plated in 96-well plates. After 24 h, these cells were treated with 0.5, 1, 5, and 10 μM YM529/ONO-5920 for 72 h. After 3 or 6 days, cell viability was assessed by the tetrazolium dye method described in Materials and methods.

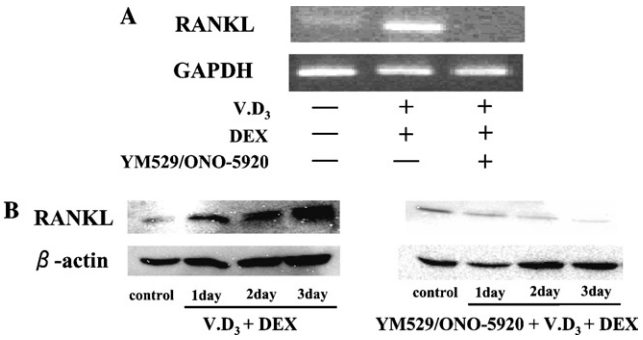

Fig. 2. Inhibition of RANKL mRNA and protein expression in ST2 cells receiving YM529/ONO-5920. ST2 cells were treated with 5 μM YM529/ONO-5920 for 24 h. These cells receiving YM529/ONO-5920 were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 72 h. (A) Photograph revealed the RT-PCR analysis for RANKL. In the $V.D_3$ and DEX group, RANKL mRNA expression was increased. YM529/ONO-5920 markedly inhibited RANKL mRNA expression. (B) ST2 cells were treated with 5 μM YM529/ONO-5920 for 24 h. These cells receiving YM529/ONO-5920 were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 1, 2, and 3 days. Photograph revealed the Western blot analysis for RANKL protein. In the $V.D_3$ and DEX group, RANKL expression was increased in a time-dependent manner from Day 1 through Day 3, whereas after YM529/ONO-5920 administration, the expression was reduced in a time-dependent manner from Day 1 through Day 3.

In the $V.D_3$ and DEX group, RANKL expression was increased in a time-dependent manner from Day 1 through Day 3, whereas after YM529/ONO-5920 administration, the expression was reduced in a time-dependent manner from Day 1 through Day 3 (Fig. 2B).

Influence of GGPP on RANKL mRNA expression in ST2 cells receiving YM529/ONO-5920

We administered YM529/ONO-5920 in combination with GGPP, a product in the mevalonate pathway, to

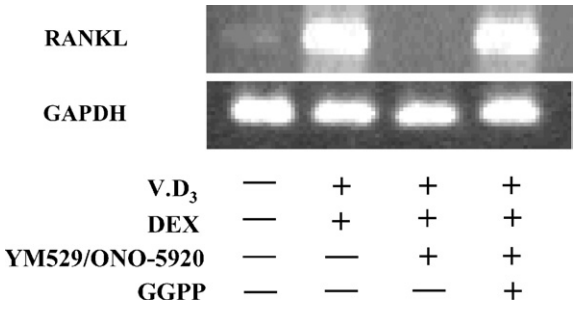

Fig. 3. RANKL mRNA expression in ST2 cells receiving YM529/ONO-5920 with GGPP. ST2 cells were pretreated with 10 μM GGPP for 4 h and then treated with 5 μM YM529/ONO-5920. After 24 h, these cells receiving YM529/ONO-5920 and GGPP were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 72 h. Photograph revealed the RT-PCR analysis for RANKL. In combination with YM529/ONO-5920 and GGPP, RANKL mRNA expression was restored to the degree observed in the $V.D_3$ and DEX group.

investigate whether or not the reduced RANKL mRNA expression in ST2 cells was due to the inhibitory action of YM529/ONO-5920 on GGPP biosynthesis exerted through its mechanism of action.

YM529/ONO-5920 alone inhibited RANKL mRNA expression, whereas in combination with GGPP, RANKL mRNA expression was restored to the degree observed in the $V.D_3$ and DEX group (Fig. 3). This suggests that the inhibition of RANKL mRNA expression in ST2 cells receiving YM529/ONO-5920 was due to the inhibition of GGPP biosynthesis.

Change in phosphorylated ERK1/2 protein in ST2 cells receiving YM529/ONO-5920

The results described above suggest that the inhibition of RANKL mRNA expression in ST2 cells receiving YM529/ONO-5920 involved the inhibition of GGPP biosynthesis in the mevalonate pathway. It is possible that the inhibition of GGPP synthesis prevented the prenylation of low-molecular G-proteins, such as Ras and Rho, thereby inhibiting signal transduction through the Ras–MEK–ERK pathway. We then investigated changes in phosphorylated ERK1/2 protein during the inhibition of RANKL expression.

In the $V.D_3$ and DEX group, the expression of phosphorylated ERK1/2 protein was higher than that of the controls from Day 1 through Day 3 (Fig. 4A). In contrast, after YM529/ONO-5920 administration, the expression was lower than that of the controls from Day 1 through Day 3 (Fig. 4B). Pretreatment with GGPP reversed the YM529/ONO-5920-induced decrease in phosphorylation of ERK (Fig. 4C). These results suggest that the inhibition of RANKL expression in ST2 cells receiving YM529/ONO-5920 is exerted by the inhibition of GGPP biosynthesis in the Ras–MEK–ERK pathway.

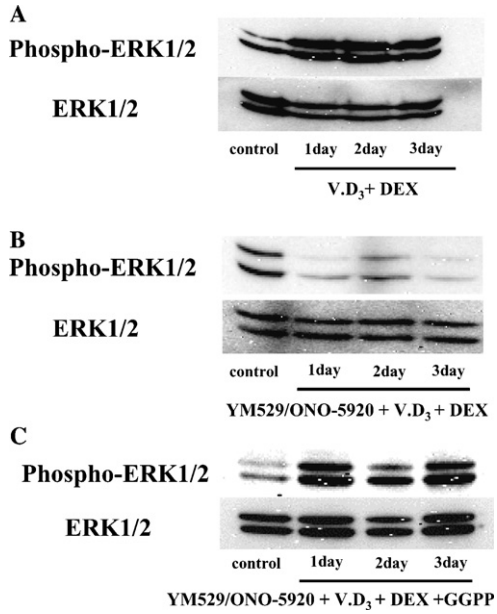

Fig. 4. Inhibition of ERK1/2 phosphorylation in ST2 cells treated with YM529/ONO-5920. ST2 cells were treated with 5 μM YM529/ONO-5920 for 24 h. These cells receiving YM529/ONO-5920 were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 1, 2, and 3 days. Whole cell lysates were generated and immunoblotted with an antibody against phosphorylated ERK1/2 (p-ERK1/2) and ERK1/2. (A) In the $V.D_3$ and DEX group, the expression of phosphorylated ERK1/2 protein was higher than that of the controls from Day 1 through Day 3. (B) After YM529/ONO-5920 treatment, the expression was lower than that of the controls from Day 1 through Day 3. (C) Pretreatment with GGPP reversed the YM529-induced decrease in phosphorylation of ERK.

Confirmation of the inhibition of RANKL mRNA and protein expression in ST2 cells using U0126

The results so far have shown that the inhibitory action of YM529/ONO-5920 on RANKL expression in ST2 cells is exerted via the inhibition of GGPP biosynthesis, and thus signal transduction in the Ras–MEK–ERK pathway. We then administered U0126 to ST2 cells to determine whether or not the inhibition of ERK1/2 would cause a decrease in RANKL mRNA and protein expression, and defined its effects on RANKL mRNA and protein expression.

Administration of U0126 reduced RANKL mRNA expression as observed with YM529/ONO-5920 (Fig. 5A). This suggests that RANKL mRNA expression is inhibited by preventing signal transduction in the MEK/ERK pathway.

We confirmed that the administration of U0126 inhibited RANKL mRNA expression in ST2 cells. We then investigated whether or not U0126 actually inhibited RANKL protein expression in ST2 cells.

As observed with YM529/ONO-5920, the administration of U0126 reduced RANKL protein expression compared with the controls in a time-dependent manner from Day 1 through Day 3 (Fig. 5B).

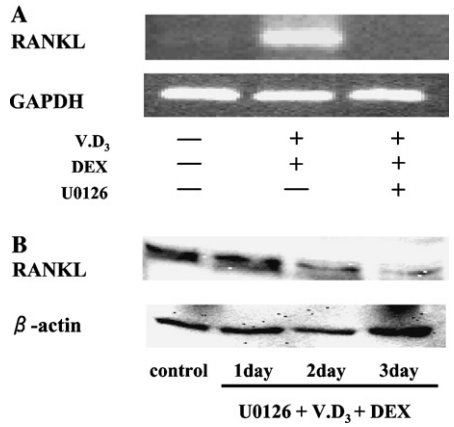

Fig. 5. Inhibition of RANKL mRNA and protein expression in ST2 cells treated with U0126. (A) ST2 cells were treated with 30 μM U0126 for 24 h. These cells receiving U0126 were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 72 h. Photograph revealed the RT-PCR analysis for RANKL as described in Materials and methods. In the $V.D_3$ and DEX group, RANKL mRNA expression was increased. U0126 markedly inhibited RANKL mRNA expression. (B) ST2 cells were treated with 30 μM U0126 for 24 h. These cells receiving U0126 were cultured in the presence of 10^{-8} M $V.D_3$ and 10^{-7} M DEX for 1, 2, and 3 days. Photograph revealed the Western blot analysis for RANKL protein. In the $V.D_3$ and DEX group, RANKL expression was increased in a time-dependent manner from Day 1 through Day 3, whereas after U0126 treatment, the expression was reduced in a time-dependent manner from Day 1 through Day 3.

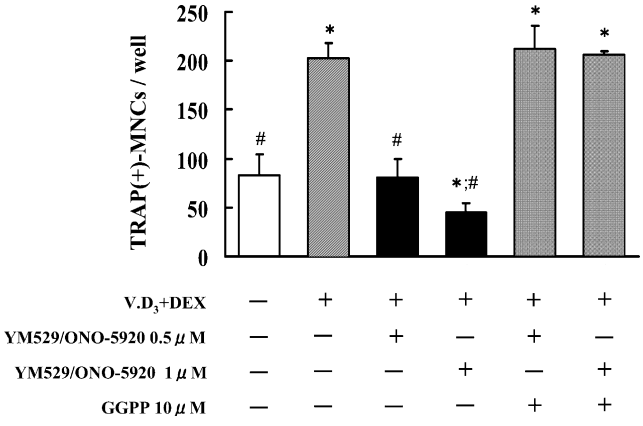

Fig. 6. Effects of YM529/ONO-5920 on the number of TRAP-positive cells in co-culture of ST2 cells and C7 cells with the potential of differentiating into osteoclasts. After culturing ST2 cells, the cells were co-cultured with C7 cells, having the potential to differentiate into osteoclasts, and TRAP-positive multinucleated cells (3 or more nuclei) were counted under a microscope after 12 days. When ST2 cells were pretreated with YM529/ONO-5920, the number of TRAP-positive cells significantly decreased in a concentration-dependent manner. Furthermore, the inhibitory effect of YM529/ONO-5920 on the induction of TRAP-positive multinucleated cells was lost by GGPP pretreatment. $^{\#}P < 0.01$, as compared with the single-treatment groups given $V.D_3$ and DEX, $^{*}P < 0.01$, as compared with the control.

Effects of YM529/ONO-5920 on the number of TRAP-positive cells in co-culture of ST2 cells and C7 cells with the potential of differentiating into osteoclasts

In C7 cells that are capable of differentiating into osteoclasts, co-culture with ST2 cells treated with vitamin D_3 + DEX significantly increased differentiation into TRAP-positive osteoclasts with 3 or more nuclei (Fig. 6). However, when ST2 cells were pretreated with YM529/ONO-5920, the number of TRAP-positive cells significantly decreased in a concentration-dependent manner. Furthermore, the inhibitory effects of YM529/ONO-5920 on the induction of differentiation of C7 cells into TRAP-positive multinucleated cells were lost by GGPP pretreatment (Fig. 6).

Discussion

In this study, we considered the possibility that YM529/ONO-5920, a newly developed nitrogen-containing bisphosphonate, has an influence on stromal cells as well and inhibits osteoclast formation by inhibiting the expression of RANKL, which plays an important role in osteoclast formation. To test this hypothesis, we investigated whether or not YM529/ONO-5920 inhibited RANKL expression in the bone marrow-derived stromal cell line of ST2 cells and defined its mechanism of action. ST2 cells expressed RANKL, which was increased by the addition of $V.D_3$ and DEX. Although $V.D_3$ and DEX have been reported to enhance RANKL expression in stromal cells, their mechanism remains unknown. According to the literature, $V.D_3$ regulates various kinds of transcription by binding to DNA after forming a heterodimer with the transcription factor, retinoid X receptor (RXR) [16]. $V.D_3$ also activates MAPK by the direct stimulation of protein kinase C (PKC) [17] or the activation of MAPK in collaboration with vitamin D receptor (VDR) and Shc protein, which is an activator of Ras [18]. DEX enhances VDR expression [16]. These reported findings raise the possibility that the increase in RANKL expression after the addition of $V.D_3$ and DEX involves transcription directly through VDR or the activation of MAPK.

We observed that YM529/ONO-5920 inhibited RANKL expression in stromal cells in the presence of $V.D_3$ and DEX, indicating that the mechanism of action is due to the inhibition of GGPP biosynthesis. Shai et al. speculated that inhibition of bone resorption by bisphosphonate is not a direct action on osteoclasts, but on osteoblasts or stromal cells. However, the details were not clear [19]. Kim et al. [20] recently reported that the inhibitory action of the bisphosphonates, alendronate and pamidronate, on bone resorption does not involve the regulation of RANKL and osteoprotegerin (OPG) expression, whereas Viereck et al. [21] reported that pamidronate increases OPG production by osteoblasts, thus indicating that the findings are still

inconsistent. Since we clearly observed the inhibitory action of a bisphosphonate on stromal cells, the action of bisphosphonates may vary in stromal cells and osteoblasts. We also observed that ERK via signals from Ras was activated by the addition of $V.D_3$ and DEX, whereas the activation was inhibited by the administration of YM529/ONO-5920. Furthermore, U0126, an MEK inhibitor, inhibited RANKL expression in ST2 cells as observed with YM529/ONO-5920. These results suggest that RANKL expression involves the MEK/ERK pathway, which is also supported by the above-mentioned $V.D_3$ action. Presently, factors known to participate in RANKL expression in ST2 cells are IL-6, NO, TGF-β1 as well as $V.D_3$ and DEX. According to the literature, IL-6, which is an accelerator of RANKL expression, activates various molecules including SHP2-Ras-MAPK and phosphoinositide 3-kinase (PI3K) through gp130, an intracellular IL-6 receptor as well as a signal transducer [22]. NO inhibits RANKL expression in ST2 cells or affects transcription by modifying the structure of transcription factors, such as c-fos, c-Jun, and NF-κB [9]. TGF-β1 inhibits RANKL expression in ST2 cells, but it enhances RANKL expression in vascular endothelial cells through CREB activation, which does not occur in ST2 cells [10,23]. These reported findings raise the possibility that transcription factors, such as c-fos and CREB, are essential to RANKL expression and that the MEK/ERK pathway, which is located upstream from these transcription factors, is involved in RANKL expression. Furthermore, since it has been reported that the inhibition of Ras activity has a substantial impact on ERK activity, but not on p38 or Akt activity [24], the inhibitory action of YM529/ONO-5920 on prenylation of Ras may be especially marked in the MEK/ERK pathway in the signaling pathways via Ras.

The above results indicate that RANKL expression in ST2 cells involves the MEK/ERK pathway and that the inhibitory action of YM529/ONO-5920 on RANKL expression in stromal cells is exerted through the inhibition of GGPP biosynthesis in the mevalonate pathway, which inhibits signal transduction in the Ras–MEK–ERK pathway. In the experiment using C7 cells, which have the potential to differentiate into osteoclasts, co-culture with ST2 cells expressing RANKL induced by $V.D_3$ + DEX resulted in differentiation into TRAP-positive multinucleated cells, but pretreatment with YM529/ONO-5920 inhibited this differentiation, and the inhibition was reversed by administration of GGPP. This finding supports our hypothesis described above.

In this study, we observed the inhibitory action of YM529/ONO-5920 on RANKL expression in the bone marrow-derived stromal cell line of ST2 cells. This finding may be a newly elucidated action of YM529/ONO-5920 in the inhibition of bone resorption and further clinical application is expected.

Acknowledgment

We thank Prof. Hayashi, Department of Immunology, School of Life Science, Faculty of Medicine, Tottori University, for providing C7 cells.

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