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Inhibition of osteoclast formation by 3-methylcholanthrene, a ligand for arylhydrocarbon receptor: suppression of osteoclast differentiation factor in osteogenic cells

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Abstract

We investigated the effects of 3-methylcholanthrene (3MC), a ligand for arylhydrocarbon receptor (AhR), on osteoclastogenesis. Osteoclast-like cells, in cocultures with mouse spleen cells and clonal osteogenic stromal ST2 cells, are formed from spleen cells by a combination of the receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) produced by ST2 cells in response to 1α,25$(OH)_2$ Vitamin D_3. 3MC dose-dependently inhibited the formation of mono- and multinuclear osteoclast-like cells. However, 3MC did not inhibit the formation of osteoclast-like cells from mouse spleen cells which was supported by the exogenous soluble RANKL and M-CSF. 3MC did not affect the formation of an actin ring and pits on slices of dentine by osteoclast-like cells, both of which are typical indices of osteoclast activity. These results suggest that 3MC affects osteoclast-supporting cells such as ST2 cells but not osteoclast precursor cells and mature osteoclastic cells. When we measured the expression levels of RANKL mRNA in ST2 cells, 3MC dose-dependently decreased the level of this mRNA. However, 3MC did not affect levels of mRNAs for osteoprotegerin (OPG), M-CSF, and the receptor of 1α,25$(OH)_2$ Vitamin D_3 in ST2 cells. Furthermore, soluble RANKL was able to counteract the inhibitory effect of 3MC on the formation of osteoclast-like cells. Our findings indicate that 3MC inhibits osteoclastogenesis *via* the inhibition of RANKL expression in osteoblastic cells.

Keywords: 3-Methylcholanthrene; AhR; RANKL; Differentiation; Osteoclast; ST2 cells

1. Introduction

The AhR has been characterized as a ligand-activated transcriptional factor induced in response to aromatic hydrocarbons, such as TCDD and 3MC. It is also a member of the bHLH/PAS (basic helix-loop-helix/Pas-Arnt-Sim) family [1–3]. TCDD and 3MC are generally considered to involve the binding of TCDD and 3MC to cytosolic AhR, and the receptor–ligand complex, in concert with an Arnt, is relocated to the nucleus, followed by sequence-specific interaction with the xenobiotic response element (XRE; CACGCT/A) of the Ah locus resulting in multigene expression [4–6]. TCDD is the most biologically toxic aromatic hydrocarbon and is classified as an environmental contaminant. When TCDD or 3MC is ingested by animals or taken up by cultured cells, these compounds are known to exert biological effects including immunotoxicity [3,7] and tumor promotion [8,9] and to induce the expression of a subclass of cytochrome P450 in liver [10]. In a previous study [11], we administrated 3MC to cultured osteoblasts and pregnant mice to clarify the effects of 3MC on the formation of bone. Consequently, 3MC inhibited the

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Abbreviations: AhR, arylhydrocarbon receptor; Arnt, AhR nuclear translocation protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 3MC, 3-methylcholanthrene; M-CSF, macrophage colony-stimulating factor; α-MEM, α-modified Eagle's medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide; OPG, osteoprotegerin; RANKL, the receptor activator of nuclear factor-κB ligand; RT-PCR, reverse transcriptase-polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TRAP, tartrate-resistant acid phosphatase; VDR, the receptor of 1α,25$(OH)_2$ Vitamin D_3.

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proliferation and differentiation of cultured osteoblastic cells, such as MC3T3-E1 and primary-cultured rat calvarial osteoblasts. Furthermore, we observed a delay in the ossification of the forelimb, hindlimb, and cervical and thoracic vertebrae in fetal mice by exposure to 3MC. By contrast, information on AhR ligands involved in osteoclastogenesis is not fully available.

Osteoclasts are multinuclear cells that are responsible for resorption of bone. The osteoclastic resorption of bone entails several processes: the development of osteoclasts from hematopoietic progenitor cells; the fusion of osteoclasts; and the secretion of acids and lysosomal enzymes into the space beneath osteoclasts [12]. The formation and activation of osteoclasts are controlled by the combined action of RANKL [13–16], OPG [17,18], and M-CSF [13] produced by osteoblasts or osteogenic stromal cells (e.g. ST2 cells). 1α,25(OH)$_2$ Vitamin D$_3$ stimulates and inhibits the expression of mRNAs for RANKL and OPG, respectively [13–18]. We used here cocultures with mouse spleen cells (osteoclast precursor cells) and ST2 cells (osteoclast-supporting cells) in a well-characterized model system that has been used for studies of osteoclast differentiation, fusion, and resorption activity [19–21]. In the present study, we examined the effects of 3MC on osteoclastogenesis and found that 3MC inhibited the formation of osteoclast-like cells *via* the inhibition of expression of RANKL by osteoclast-supporting cells, ST2 cells.

2. Materials and methods

2.1. Materials

3MC and 1α,25(OH)$_2$ Vitamin D$_3$ were purchased from Sigma. Sodium tartrate was purchased from Wako Pure Chemical Industries Ltd. α-MEM, penicillin/streptomycin antibiotic mixture, and fetal bovine serum were purchased from Invitrogen.

2.2. Formation of osteoclast-like cells in vitro

Spleen cells were collected from splenic tissues of 6–9-week-old male ddY mice. Erythrocytes contaminating the spleen cell fraction were eliminated by adding 0.83% ammonium chloride in 10 mM Tris–HCl (pH 7.4) to the cell pellet. Mouse clonal stromal cells from bone marrow, ST2 cells, were supplied by the RIKEN Cell Bank. ST2 cells were cocultured (2×10^4 cells/well) with mouse spleen cells (1×10^5 cells/well) in α-MEM that contained 10% fetal bovine serum and 10^{-8} M 1α,25(OH)$_2$ Vitamin D$_3$ in 48-well plates (0.98 cm^2/well) [21]. 3MC was dissolved in dimethylsulfoxide (final concentration of 0.05%) and 0.05% dimethylsulfoxide did not affect the formation of osteoclast-like cells. The cultures were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. Fresh medium and 3MC were supplied at 2-day intervals. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

Spleen cells (5×10^4 cells/well) in 48-well plates were cultured with 50 ng/mL soluble human RANKL and 30 ng/mL M-CSF for 5 days. Furthermore, the murine monocytic cell line RAW264.7 was used for the formation of osteoclast-like cells. RAW264.7 cells (1×10^4 cells/well) in 48-well plates were exposed to 10^{-5} M PD98059 (Promega Corp) and 100 ng/mL soluble human RANKL (PeproTech EC Ltd) and cultured for 3 days [22]. Fresh medium and 3MC were supplied at 2-day intervals.

2.3. Localization of tartrate-resistant acid phosphatase (TRAP) and F-actin

After coculture for 8 days, adherent cells were fixed in 3.6% formaldehyde for 5 min and then in a mixture of ethanol and acetone (1:1, v/v) for 1 min. Then they were stained for TRAP activity, as previously described [21]. TRAP has been extensively used as a cell specific marker for osteoclasts, although its function has remained obscure. TRAP-positive mononuclear cells and TRAP-positive multinuclear cells (with three or more nuclei) were counted under a microscope (IX70; Olympus). For identification of F-actin, osteoclast-like cells were exposed to 10^{-6} M 3MC for 2 days after coculture for 6 days, and then formaldehyde-fixed cells were stained with 2 units of Alexa Fluor 546 phalloidin (Molecular Probes, Inc.).

2.4. Quantitation of bone resorption

The formation of pits on slices of dentine was monitored to determine the capacity for bone resorption of the osteoclast-like cells formed [23]. Bone marrow cells and ST2 cells were cocultured for 6 days in collagen-gel-coated dishes (55 cm^2). The cocultures were treated with 0.2% collagenase (Wako Pure Chemical Industries) at 37° for 20 min to detach osteoclast-like cells from the dishes. The osteoclast-like cells were plated on dentine slices (4 mm in diameter), which had been placed in 96-well plates (0.48 cm^2/well) that contained 0.2 mL of α-MEM supplemented with 10% fetal bovine serum. After incubation for 48 hr, the cells were removed from the dentine slices, and then resorption pits that had been formed were stained with Mayer's hematoxylin. The number and the total area of pits were observed under a microscope (Olympus).

2.5. Cell viability

We used 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT; Dojindo) to examine the viability of ST2, mouse spleen, and RAW264.7 cells. These cells (1×10^4/well; 96-well plates) were subcultured with 3MC at various concentrations for the indicated times.

The cells were treated with MTT (50 μg/well) and then absorbance at 570 nm was measured.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting analysis

We detected mRNAs for AhR, Arnt, RANKL, OPG, M-CSF, and the receptor of VDR by RT-PCR–Southern blotting. Total RNA was extracted from ST2 cells, spleen cells, and mature osteoclast-like cells by the acid guanidinium-phenol-chloroform method [24]. Total RNA (2 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase, Superscript (200 units; Promega Corp), with random primers (50 ng) in a 25-μL reaction mixture. cDNA was amplified in 20 μL of Taq DNA polymerase mixture (Takara) that contained 1 μM sense primer, 5′-GGGATTGATTTTGAAGATATCAG-3′, and 1 μM antisense primer, 5′-AATGCCTGAGAACCTGGAATTTC-3′, for mouse AhR (accession number D38417, 2536–3317: 782 bp); 1 μM sense primer, 5′-GCTATAATCATTCCCAGGTTTCT-3′, and 1 μM antisense primer, 5′-CATTGTTGTAGGTGTTGCTTTGG-3′, for mouse Arnt (accession number NM012780; 1603–2367, 765 bp); 1 μM sense primer, 5′-CAGGTTTGCAGGACTCGAC-3′, and 1 μM antisense primer, 5′-AGCAGGGAAGGGTTGGACA-3′, for mouse RANKL (accession number AF013170, 434–1034: 601 bp); 1 μM sense primer, 5′-CCACTCTTATACGGACAGCT-3′, and 1 μM antisense primer, 5′-TCTCGGCATTCACTTTGGTC-3′, for mouse OPG (accession number U94331, 291–796: 506 bp); 1 μM sense primer, 5′-TTGCCAAGGAGGTGTCAGAA-3′, and 1 μM antisense primer, 5′-TATTGGAGAGTTCCTGGAGC-3′, for mouse M-CSF (accession number M21952, 251–511: 261 bp); 1 μM sense primer, 5′-TCTGAGGAGCAACAGCACAT-3′, and 1 μM antisense primer, 5′-TCACCTCAATGGCACTTGAC-3′, for mouse VDR (accession number D31969, 481–904: 424 bp); or 1 μM sense primer, 5′-ACTTTGTCAAGCTCATTTCC-3′, and 1 μM antisense primer, 5′-TGCAGCGAACTTTATTGATG-3′, for mouse GAPDH (accession number M32599, 957–1223: 267 bp). Each reaction cycle, performed 35, 35, 24, 24, 19, 20, and 19 times for amplification of the cDNA for AhR, Arnt, RANKL, OPG, M-CSF, VDR and GAPDH, respectively, consisted of incubation at 94° for 30 s, 60° for 30 s (51° for AhR), and 72° for 60 s. The products of PCR were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA marker fragments (molecular weight marker V; Boehringer Mannheim) were used as size markers.

For quantitative analysis of mRNAs for RANKL, OPG, M-CSF, VDR, and GAPDH, the products of PCR were blotted onto nylon membranes (MagnaGraph; Micron Separation Inc.) after electrophoresis. The blots were prehybridized at room temperature for 2 hr in 6× SSPE (1× SSPE consists of 0.15 M NaCl, 8.65 mM $NaH_2PO_4{\cdot}2H_2O$, and 1.25 mM EDTA, pH 7.4) that contained 2× Denhardt's solution (1× Denhardt's solution consists of 0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 50% formamide, and 0.5% SDS. Then the blots were allowed to hybridize at 42° for 16 hr in the same solution with a ^{32}P-labeled cDNA probe specific for RANKL, OPG, M-CSF or GAPDH at 1×10^6 cpm/mL. The blots were washed twice in 0.1× SSC and 0.1% SDS at 60° for 1 hr and then were analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film).

3. Results

3.1. Inhibitory effects of 3MC on formation of osteoclasts

3MC binds to AhR in the cytosol and the receptor–ligand complex, together with Arnt, is located to the nucleus. We utilized a coculture system that consisted of mouse spleen cells and mouse osteogenic stromal cells, ST2 cells, for inducting the formation of osteoclast-like cells. We first performed RT-PCR analysis using specific primers for the amplification of mouse genes for AhR and Arnt, as part of our effort to characterize the effects of 3MC in the ST2 cells, spleen cells, and osteoclasts formed. Figure 1 shows that transcripts specific for AhR and Arnt can be detected in the cells used in this study.

We demonstrated that the formation of TRAP-positive mononuclear (A) and multinuclear (B) osteoclast-like cells

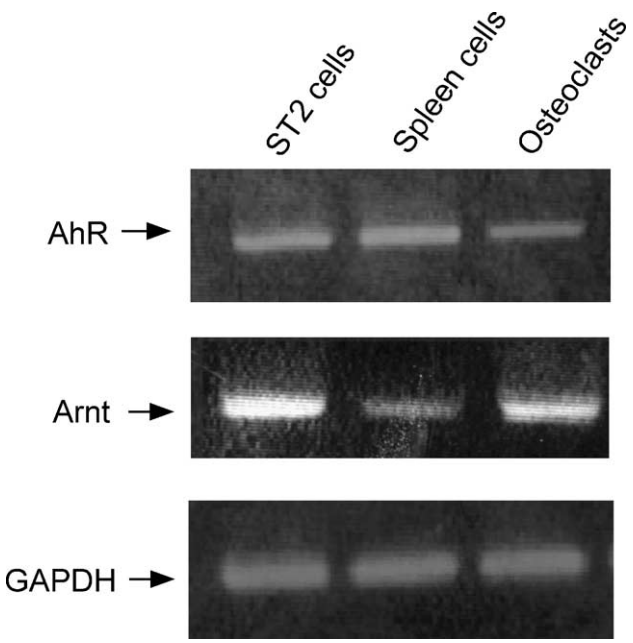

Fig. 1. Detection of transcripts specific for AhR and Arnt in ST2 cells, mouse spleen cells, and mature osteoclasts by RT-PCR. Total RNA was isolated from each cell and the levels of mRNA for mouse AhR (782 bp) and mouse Arnt (768 bp) in the respective cells were examined by RT-PCR with specific primers (see text for details). Mature osteoclasts were formed in cocultures with mouse spleen cells and ST2 cells. GAPDH mRNA was used as an internal control.

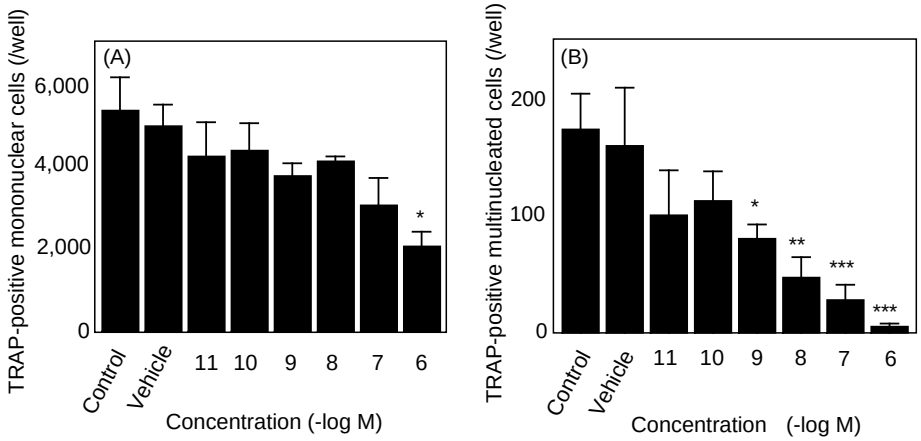

Fig. 2. Inhibition of formation of TRAP-positive osteoclast-like cells by 3MC. Mouse spleen cells were cocultured with ST2 cells in 48-well plates for 8 days in α-MEM that contained 10% fetal bovine serum and 10^{-8} M $1\alpha,25(OH)_2D_3$ with and without 3MC at the indicated concentrations. Control cultures (Vehicle) were supplemented with 0.05% dimethylsulfoxide. Fresh medium with and without 3MC were supplied at 3-day intervals. The cells were stained for TRAP activity, and TRAP-positive mononuclear cells (A) and TRAP-positive multinuclear cells (B) were counted under a microscope. Data are means ± SE of results from four determinations. $^{*}P < 0.05$ vs. controls; $^{**}P < 0.01$ vs. controls; $^{***}P < 0.001$ vs. control. Bars = 1 mm.

was inhibited by the addition of 3MC to the culture medium in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$ (Fig. 2). Inhibition of the formation of multinuclear osteoclast-like cells was observed upon addition of 10^{-11} M 3MC, and 10^{-6} M 3MC completely inhibited this process. Next, we determined the target cells of 3MC. When we formed osteoclast-like cells from mouse spleen cells by the addition of 30 ng/mL M-CSF and 50 ng/mL human recombinant soluble RANKL to the culture medium, 3MC did not inhibit the formation of osteoclast-like cells in this system (Fig. 3A). Furthermore, 3MC did not affect the formation of osteoclast-like cells from murine monocytic RAW264.7 cells exposed to 100 ng/mL human recombinant soluble RANKL (Fig 3B). These results show that 3MC functions in osteogenic ST2 cells.

We also confirmed, using MTT, that the effects of 3MC were not attributable to its generalized toxicity to ST2, spleen, and RAW264.7 cells during our experimental periods (Fig. 4).

3.2. Effects of 3MC on osteoclast activity

The effects of 3MC on the resorption activity of osteoclast-like cells were examined. The osteoclast-like cells formed from spleen cells were exposed to 10^{-6} M 3MC for 2 days. 3MC did not affect the formation of an actin ring

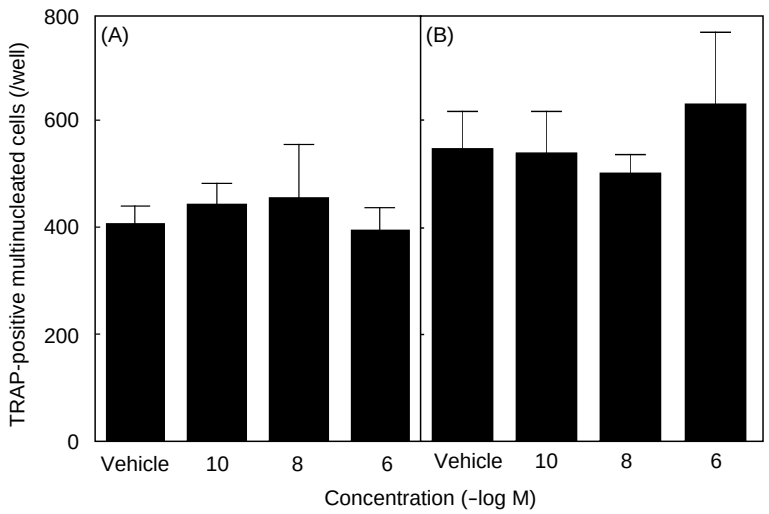

Fig. 3. Effects of 3MC on formation of osteoclast-like cells. (A) Mouse spleen cells were cultured with 30 ng/mL M-CSF and 50 ng/mL soluble RANKL in 48-well plates for 5 days in α-MEM that contained 10% fetal bovine serum with and without 3MC at the indicated concentrations. Control cultures (Vehicle) were supplemented with 0.05% dimethylsulfoxide. Fresh medium with and without 3MC were supplied at 2-day intervals. The cells were stained for TRAP activity, and TRAP-positive multinuclear cells were counted under a microscope. (B) RAW264.7 cells derived from leukemic mouse monocytes were supplied by the RIKEN Cell Bank. Cells were cultured with 100 ng/mL soluble RANKL and 10^{-5} M PD98059 in 48-well plates for 5 days in α-MEM that contained 10% fetal bovine serum with and without 3MC at the indicated concentrations. Data are means ± SE of results from four determinations.

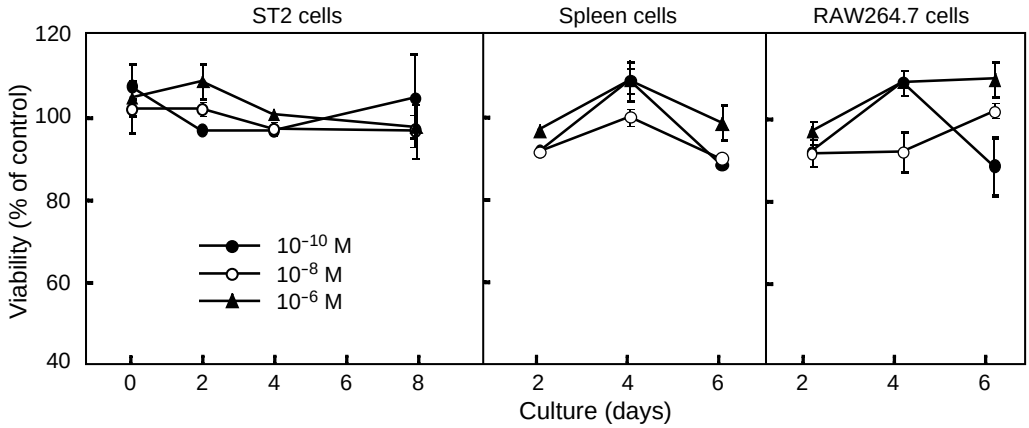

Fig. 4. Effects of 3MC on cell viability. ST2, spleen (with 30 ng/mL M-CSF), and RAW264.7 cells were cultured in 96-well plates for the indicated periods in α-MEM that contained 10% fetal bovine serum and 3MC at various concentrations. Cells were treated with MTT and then absorbance at 570 nm was measured. Data are means ± SE of results from three determinations.

(Fig. 5A) and pits on dentin slices (Fig. 5B), both of which are typical indices of osteoclast activity.

3.3. Mechanism of action of 3MC

To characterize the mode of action of 3MC, we examined the expression level of mRNAs for RANKL, OPG, M-CSF, and VDR in ST2 cells by RT-PCR–Southern blotting. ST2 cells were treated with 3MC at 10^{-9} to 10^{-6} M in the presence of 10^{-8} M 1α,25$(OH)_2D_3$ for 2 days and we detected a decreased expression level of mRNA for RANKL at 10^{-8} M 3MC (Fig. 6A). By contrast, 3MC had no effect on the level of mRNAs for OPG, M-CSF, and VDR (Fig. 6A). When ST2 cells were treated with 10^{-6} M 3MC for 2 and 5 days in the presence of 10^{-8} M 1α,25$(OH)_2D_3$, the expression level of mRNA for RANKL was also inhibited (Fig. 6B). To examine that inhibition of expression of RANKL mRNA by 3MC is critical in

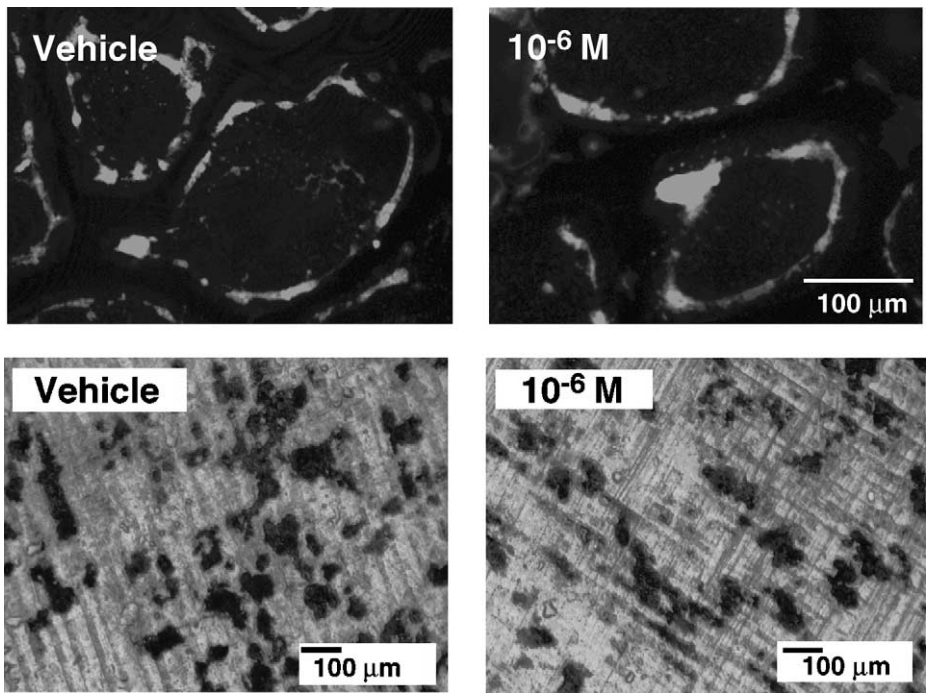

Fig. 5. Effects of 3MC on formation of an actin ring (A) and pits on slices of dentine (B) by osteoclast-like cells. Staining for actin; TRAP-positive multinuclear cells that had been formed by coculture with spleen cells and ST2 cells for 8 days were fixed and stained with Alexa Fluor 546 phalloidin. Pit assay (see text for details); osteoclast-like cells were cultured in 96-well plates (0.48 cm^2/well) and a slice of dentine was placed in each well. 3MC was supplied to osteoclast-like cells on each dentine slice at 10^{-6} M. After culture for 48 hr, adherent cells were removed from the slices of dentine, and resorption pits that formed on the surface of dentine were stained with Mayer's hematoxylin. Arrow shows the pits on dentine slices. Bar, 100 μm. Data are representative of results from three separate experiments.

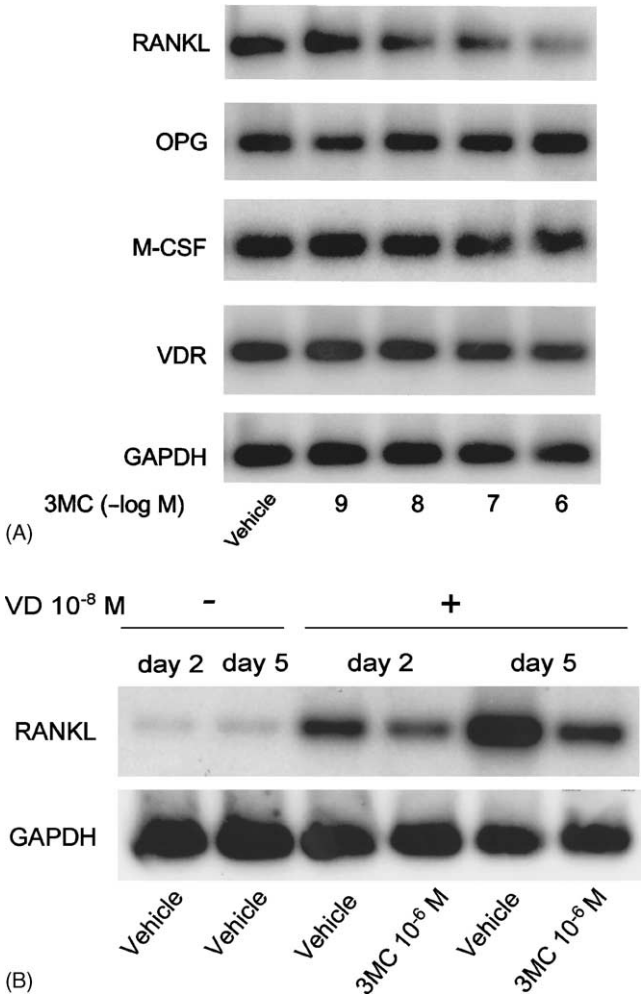

Fig. 6. Expression of mRNAs for RANKL, OPG, M-CSF, and VDR in ST2 cells exposed to 3MC. Total RNA was isolated from ST2 cells that had been treated for 2 days with 3MC at the indicated concentrations in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$ (A) and treated with 10^{-6} M 3MC for 2 and 5 days in the presence of 10^{-8} M $1\alpha,25(OH)_2D_3$ (B). We performed RT-PCR using specific primers for RANKL, OPG, M-CSF, VDR and GAPDH. The RT-PCR products were subjected to electrophoresis in a 2% agarose gel and were allowed to hybridize with ^{32}P-labeled cDNAs for RANKL, OPG, M-CSF, VDR and GAPDH, as described in Section 2. The results shown are representative of the results of three experiments.

the formation of osteoclast-like cells, we added soluble RANKL to the medium of 10^{-6} M 3MC-treated coculture system. As shown in Fig. 7, addition of soluble RANKL to the medium attenuated the inhibition of osteoclast-like cell formation by 3MC, and the effect of soluble RANKL was dose-dependent. 100 ng/mL soluble RANKL rescued 70% of the formation of osteoclast-like cells that was inhibited by 10^{-7} M 3MC.

3.4. Effects of AhR antagonist, resveratrol, on 3MC-induced inhibition of formation of osteoclast-like cells

Resveratrol is a polyphenol from plant that functions as an AhR antagonist [25]. As shown in Fig. 8, the

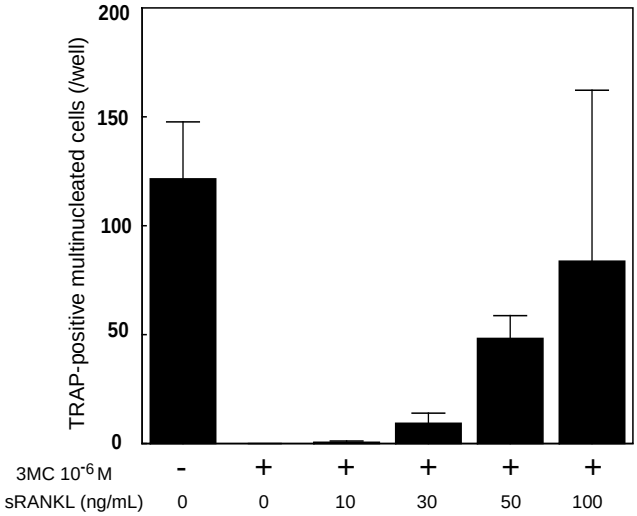

Fig. 7. The effects of soluble RANKL on the formation of TRAP-positive osteoclast-like cells in cocultures exposed to 3MC. Mouse spleen cells were cocultured with ST2 cells in 48-well plates for 8 days in α-MEM that contained 10% fetal bovine serum and 10^{-8} M $1\alpha,25(OH)_2D_3$ with and without 10^{-7} M 3MC and soluble RANKL (sRANKL) at various concentrations. Fresh medium and soluble RANKL were supplied at 3-day intervals. The cells were stained for TRAP activity and TRAP-positive multinuclear cells were counted under a microscope. Data are means ± SE of results from three determinations.

inhibition of the formation of osteoclast-like cells by 3MC in a coculture system was antagonized by resveratrol in a dose-dependent manner. 10^{-6} M resveratrol rescued 40% of the formation of osteoclast-like cells that was inhibited by 10^{-8} M 3MC. The addition of resveratrol (10^{-8} to 10^{-5} M) alone did not inhibit the formation of osteoclast-like cells.

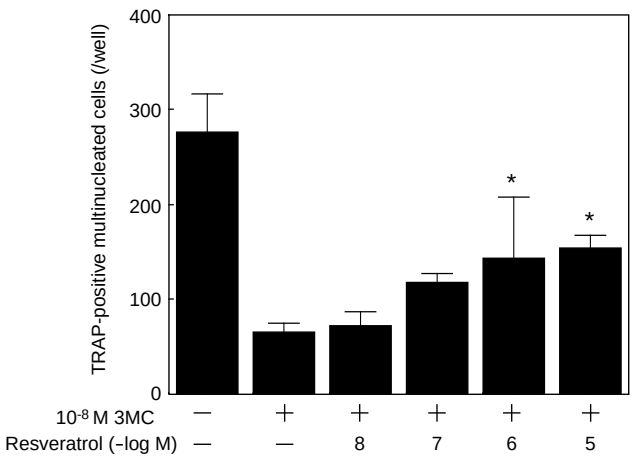

Fig. 8. Effects of resveratrol on 3MC-induced inhibition of osteoclast formation. Spleen cells were cocultured with ST2 cells in 48-well plates for 8 days in α-MEM that contained 10% fetal bovine serum, 10^{-8} M $1\alpha,25(OH)_2D_3$, and resveratrol at various concentrations in the presence and absence of 10^{-8} M 3MC. Fresh medium and 3MC were supplied at 3-day intervals. The cells were stained for TRAP activity, and TRAP-positive multinuclear cells were counted under a microscope. Data are means ± SE of results from three determinations. $^{*}P < 0.05$ vs. 10^{-8} M 3MC in the absence of resveratrol.

4. Discussion

The effect of exposure to environmental endocrine disruptors on bone metabolism in mammals is not well understood. Therefore, we attempted to clarify the potential effects of AhR ligands, which have the most toxic effects on animal, on bone metabolism. Bone modeling and remodeling involve the complex and tightly coordinated actions of bone-forming osteoblasts and bone-resorbing osteoclasts. In a previous study [11], we found that 3MC, a ligand of AhR, inhibits not only cell proliferation but also the differentiation of cultured osteoblasts. Furthermore, we showed that injection of 3MC into pregnant dams causes abnormalities and a delay in ossification of bones in fetuses. Gierthy *et al*. also reported that TCDD inhibits the differentiation of rat osteoblasts [26]. Thus, AhR ligands might be critical compounds for osteoblast metabolism. However, little information on the effects and mechanism of action of AhR ligands in osteoclastogenesis is available, when compared with the information on those in osteoblastogenesis. Therefore, we examined the effects of 3MC on osteoclastogenesis in the present study.

We demonstrated that 3MC inhibited the differentiation and fusion of osteoclasts but did not affect the resorption activity of osteoclasts. Furthermore, we found that 3MC acts directly on osteoclast-supporting cells such as ST2 cells but not on osteoclast precursors. In an effort to clarify the mode of action of 3MC in osteoclastogenesis, we focused on the expression of mRNAs for RANKL, OPG, and M-CSF in ST2 cells that support the formation of osteoclast-like cells, and found that 3MC inhibited the expression of mRNA for RANKL but did not affect that for OPG or M-CSF. Furthermore, the inhibitory effects of 3MC on osteoclastogenesis could reproduce by using mouse calvarial primary osteoblasts instead of ST2 cells (data not shown). RANKL is a membrane-bound protein that induces the formation of osteoclast-like cells in combination with M-CSF [13–16]. By contrast, OPG, which is a decoy receptor for RANKL, inhibits the formation of osteoclast-like cells [17,18]. The RANKL and OPG produced by osteoblasts or osteogenic stromal cells are key regulators of osteoclastogenesis. The ratio of the level of RANKL to that of OPG in the microenvironment is critical in the regulation of the formation of osteoclast-like cells. In fact, the addition of soluble RANKL to the medium of 3MC-treated coculture system attenuated, in large part, the inhibition of osteoclast-like cell formation by 3MC. Our findings suggest that a low level of RANKL that is expressed in ST2 cells in response to 3MC might attenuate the formation of osteoclast-like cells.

Our previous study showed that 3MC inhibits the proliferation and differentiation of osteoblasts, such as primary rat calvarial osteoblastic cells and mouse preosteoblastic cell line MC-3T3E1 [11]. The inhibitory effective doses of 3MC for the proliferation and differentiation (ALPase activity, osteocalcin expression, and the deposition of calcium) of osteoblasts were 10^{-6} and 10^{-7} M, respectively. By contrast, we found in this study that the exposure of the cells to 10^{-9} and 10^{-8} M 3MC inhibited the formation of osteoclast-like cells and the expression of mRNA for RANKL, respectively. Thus, the comparison of the effective doses of 3MC on cultured cells shows that it inhibits osteoclastogenesis stronger than osteoblastogenesis.

AhR has been found in the cytosol of cells of almost all mammalian organs and tissues. We detected mRNAs for AhR and Arnt by RT-PCR using our coculture model system including ST2 cells, spleen cells, and osteoclasts. Therefore, it seems possible that 3MC might affect the formation of osteoclast-like cells through signals carried by AhR and Arnt in ST2 cells. Resveratrol has been reported to inhibit the effects of TCDD on bone formation [25]. We also confirmed that it partially inhibited the effects of 3MC on the formation of osteoclast-like cells. Thus, our study suggests that AhR signals in osteogenic stromal cells might have a critical effect on the formation of osteoclast-like cells.

The AhR ligand complex is known to bind to the XRE (CACGCT/A) that is located in the 5′-flanking regions of the target genes and such binding results in the expression of multiple genes [4–6]. Therefore, we searched the sequence database of the 5′-flanking region of the mouse gene for RANKL and found that there is one XRE (CACGCT; −614 to −619) in the 5′-flanking region of the mouse gene for RANKL [27]. We obtained the 5′-flanking region (+51 to −918) of the mouse gene for RANKL [27] and nested deletion mutants by RT-PCR. However, a preliminary transient transfection study using a luciferase reporter gene showed that a XRE sequence (−614 to −619) in the RANKL gene might not affect the transcriptional activity of 3MC (data not shown). The sequences upstream of the 5′-flanking region of the RANKL gene may need to inactivate the expression of this gene.

The mechanism of the 3MC-induced alteration of RANKL expression in ST2 cells is as yet unknown. To date, it has been noted that there are similarities between the dioxin-induced suppression of osteoblastic differentiation and the dioxin-mediated alterations in the response to 17β-estradiol [26]. Namely, TCDD inhibits estrogen-induced cathepsin D gene expression [28] and estrogen-induced responses in the rodent uterus [29] and mammary gland [30]. By contrast, Swanson and coworkers described that the AhR ligand complex interacts with estrogen receptors or estrogen response elements, and induces the antiestrogenic activity of TCDD [31,32]. Very recently, Ohtake *et al*. have reported that dioxins can mimic the effects of estrogens through a mechanism that involves the activation of estrogen receptors by a transcriptionally active AhR–Arnt complex [33]. Under such circumstances, the action of 3MC on ST2 cells might occur, in part,

through the estrogen receptor-dependent pathways. However, the direct regulation of RANKL by estrogen in osteoblastic cells has not yet been shown [34]. Further investigations are needed to examine these possibilities.

Our findings indicate that the AhR ligand 3MC inhibits the formation of osteoclast-like cells through the inhibition of the expression of RANKL in osteoclast-supporting cells. Further research on AhR ligands might provide a clue to the development of tools useful in the prevention and treatment of postmenopausal osteoporosis.

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