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Thiazolidinediones Inhibit Osteoclast-Like Cell Formation and Bone Resorption *in Vitro**

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ABSTRACT

Osteoblasts and adipocytes are derived from common bone marrow stromal cells that play crucial roles in the generation of osteoclasts. Activation of peroxisome proliferator-activated receptor-γ (PPARγ) induces adipogenic differentiation of stromal cells; however, whether this would affect osteoblast/osteoclast differentiation is unknown. Thus, we examined the effects of the thiazolidinedione (TZD) class of antidiabetic agents that activate PPARγ on osteoblast/osteoclast differentiation using mouse whole bone marrow cell culture. As reported, all TZDs we tested (troglitazone, pioglitazone, and BRL 49653) markedly increased the number of Oil Red O-positive adipocytes and the expression of adipsin and PPARγ 2. 1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] did not affect adipogenic differentiation induced by TZDs. TZDs did not affect alkaline phosphatase activity, an early marker of osteoblastic differentiation, despite their marked adipogenic effects. TZDs decreased the number of tartrate-resistant acid phosphatase-positive multinucleated osteoclast-like cells induced by 1,25-$(OH)_2D_3$ or PTH. Troglitazone dose dependently inhibited basal and 1,25-$(OH)_2D_3$- and PTH-induced bone resorption as assessed by pit formation assay. Interleukin-11 blocked the induction by troglitazone of adipogenesis, but had no effect on the inhibition of osteoclast-like cell formation. These results indicate that TZDs are potent inhibitors of bone resorption in vitro. Inhibitory effects of TZDs on osteoclastic bone resorption was not osteotropic factor specific and did not appear to be related to their adipogenic effects. Thus, TZDs may suppress bone resorption in diabetic patients and prevent bone loss. (*Endocrinology* **140:** 5060–5065, 1999)

IN THE BONE marrow, osteoblasts and adipocytes are derived from common precursor cells that also give rise to chondroblasts, myoblasts, and fibroblasts (1–4). These mesenchymal stem cells or stromal cells also play crucial roles in the generation of bone-resorbing osteoclasts from hemopoietic stem cells (5). Because bone loss is associated with an increase in bone marrow adipose tissue in many conditions such as aging (6, 7), promotion of adipogenic differentiation could affect osteoblastic and/or osteoclastic differentiation from respective bone marrow progenitor cells. However, the relationship between adipogenesis and osteogenesis is not fully understood.

Peroxisome proliferator-activated receptor-γ (PPARγ), a member of the steroid receptor superfamily, plays a pivotal role in the differentiation of adipocytic cells (8). PPARγ activation by 15-deoxy-$\Delta^{12,14}$-PG J2 or the thiazolidinedione (TZD) class of antidiabetic agents leads to adipogenic differentiation of various types of cells (8–12). Because bone marrow tissue and bone marrow cells express PPARγ, it is plausible that TZDs, such as troglitazone (Tro), pioglitazone (Pio), and BRL 49653 (BRL), affect bone marrow cell differentiation. Indeed, Gimble *et al.* reported that TZDs promote adipogenesis in a mouse bone marrow stromal cell line, BMS2, and primary mouse bone marrow cells (13). Furthermore, forced expression of PPARγ in fibroblasts or myoblasts induce transdifferentiation of these cells into adipocytes (11, 14). These results suggest that TZDs affect osteoblastic differentiation of bone marrow stromal cells. Subsequently, they also may affect osteoclastic differentiation. However, little is known about the effects of TZDs on the differentiation of osteoblasts and osteoclasts.

TZDs are clinically useful for diabetic patients, who are commonly associated with increased bone resorption (15) and/or high risk of osteoporosis, such as postmenopausal state and aging. Thus, it is important to clarify if adipogenic actions of TZDs in bone marrow affects bone resorption and formation. We examined the effects of TZDs on osteoblastic and osteoclastic differentiation using mouse whole bone marrow cell culture and showed an inhibitory effect of TZDs on osteoclast-like cell formation and bone resorption *in vitro*.

Materials and Methods

Reagents

Cell culture medium and supplements were purchased from Life Technologies, Inc. (Rockville, MD). FBS was obtained from JRH Bioscience (Lenexa, KS). Tro was a gift from Sankyo Co., Ltd. (Tokyo, Japan). Recombinant human interleukin-11 (rhIL-11) was obtained from Genzyme Co. (Cambridge, MA). The other commercially available agents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

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Assay of tartrate-resistant acid phosphatase-positive multinucleated cell [TRAP(+)MNC] formation

TRAP(+)MNC formation was assessed as described previously with modification (16). Bone marrow cells were disaggregated from tibia and femur of 6-week-old male ddY mice (Japan SLC, Shizuoka, Japan). Cells were plated in 24-well plates in αMEM supplemented with 10% FBS. Twenty-four hours later, medium was changed, and test agents were added to the culture. After 3 days, medium was changed again, and the cells were cultured 3 more days in the presence of test agents. At the end of culture, cells were fixed with 10% formalin and stained with TRAP. All TRAP-positive cells with three or more nuclei in each well were counted. All experiments were performed in quadruplicate. All procedures involving animals were approved by institutional animal care committee.

Pit formation assay on dentine slice

The pit formation assay was performed as previously described (17) with modification. Briefly, bone marrow cells disaggregated from hind limbs of 14-day-old ICR mice were plated on a dentine slice in DMEM supplemented with 10% FBS. Four hours later, cells were rinsed once with the medium and cultured with test agents for 4 days. At the end of the culture, cells were subjected to cell viability assay, and the dentine slices were subjected to pit formation assay. For cell viability assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to the culture at a final concentration of 25 μg/ml and incubated for 4 h. After the incubation, media were removed, the precipitated dye was solubilized into dimethylsulfoxide, and absorbance was measured at 540 nm. Dentine slices were cleaned with a handy engine (Yoshida Seiko Co. Ltd, Tokyo, Japan) for 10 sec and stained with acid hematoxylin. The total area of the pits was measured by an image analyzer (PIAS-LA555, PIAS Co. Ltd, Tokyo, Japan).

Alkaline phosphatase assay

Bone marrow cells were plated in 12-well plates and cultured as described for the TRAP(+)MNC assay. At the end of culture, cells were washed twice with ice-cold PBS and scraped in 10 mM Tris-HCl containing 2 mM $MgCl_2$ and 0.05% Triton X-100, pH 8.2. The cell suspension was homogenized using Pellet Pestle (Kontes, Vineland, NJ) on ice after two cycles of freezing and thawing. Aliquots of supernatants were subjected to protein assay using a Bio-Rad Laboratories, Inc. kit (Richmond, CA) according to Bradford's method and to alkaline phosphatase (ALP) activity measurement as described previously (18).

Oil Red O staining

Bone marrow cells were cultured as described above. Cells were fixed in 10% formaldehyde for 10 min, then in 60% isopropanol for 1 min, stained with Oil Red O for 30 min, and rinsed briefly with 60% isopropanol. All Oil Red O-positive cells in each well were counted.

RT-PCR

Bone marrow cells were cultured as described above in 6-cm plates. Total RNA was isolated using Isogen (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instruction. The resultant RNA samples were further purified by a round of lithium chloride (2 M) precipitation, followed by a round of ethanol precipitation. The amount of RNA was calculated with the absorbance at 260 nm, and all the samples were diluted to the same concentration (0.1–0.2 μg/μl). One microgram of the diluted RNA samples was electrophoresed on a 1% agarose gel, stained with ethidium bromide (0.5 μg/ml), and visualized by UV transilluminator. The integrity and equality of RNA samples were verified by the band intensity of ribosomal RNA 28S and 18S. RT-PCR was performed as described with modification (19). One microgram of RNA was reverse transcribed by incubation for 5 min at room temperature and then for 90 min at 42 C with 100 U Moloney murine leukemia virus transcriptase (Life Technologies, Inc.); 5 mM random hexamer (Roche Molecular Biochemicals, Indianapolis, IN); 2.5 μM oligo(deoxythymidine)$_{16}$ (Roche Molecular Biochemicals); 1 U/μl ribonuclease inhibitor (Promega Corp., Madison, WI); 1 mM each of deoxy (d)-ATP, dCTP, dGTP, and dTTP; and 1 × *Taq* reaction buffer (Promega Corp.) supplemented with 5 mM $MgCl_2$ in a total volume of 20 μl. After the reaction, the mixture was heated for 5 min at 95 C and diluted to 60 μl with 1 × *Taq* buffer. Three microliters of the products were used for PCR amplification in a total volume of 20 μl containing 1 × *Taq* reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, and 0.5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer Corp., Norwalk, CT). The primers used were 5′-gtgaaccactgatattcagg-3′ and 5′-ctgatgcactgcctatgagc-3′ for PPARγ1, 5′-gggtcagctcttgtgaatgg-3′ and 5′-ctgatgcactgcctatgagc-3′ for PPARγ2, 5′-tcttgatttacacggaggtg-3′ and 5′-tcttgtttgtttgtccagtg-3′ for lipoprotein lipase (LPL), 5′-atacgaggacaaacaagtgg-3′ and 5′-gtaaccacaccttcgagtg-3′ for adipsin, 5′-aaacacacgaactgcagcac-3′ and 5′-tcttcttcccaggcaggctc-3′ for osteoprotegerin (OPG) or osteoclastogenesis inhibitory factor, and 5′-tcttcagctgatggtgtatg-3′ and 5′-gagtctcagtctatgtcctg-3′ for osteoclast differentiation factor (ODF) or OPG ligand. PCR cycles were performed in GeneAmp PCR system 2400 (Perkin-Elmer Corp.) with the following temperature profile: denaturation at 95 C for 30 sec, and primer annealing and primer extension at 60 C, each for 30 sec. After initial denaturation (9 min), the cycle was repeated 20–45 times, followed by a final extension step of 10 min at 60 C. Half of the PCR product was electrophoresed on a 2.5% NuSieve 3:1 (FMC BioProducts, Rockland, ME) agarose gel and stained with 0.5 μg/ml ethidium bromide, and bands were visualized by UV transilluminator. The identity of PCR products was confirmed by fluorescence-based dideoxy sequencing of each PCR product using ABI PRISM 310 genetic analyzer (Perkin-Elmer Corp.).

Statistical analyses

All statistical analyses were performed using StatView software (version 4.5, Abacus Concepts, Inc., Berkeley, CA). The results were analyzed with one-way ANOVA followed by Bonferroni/Dunn's test. $P < 0.05$ was considered significant.

Results

Effects of TZDs on osteoblastic and adipocytic differentiation

To determine whether PPARγ activation in bone marrow stromal cells suppress osteoblastic differentiation, we measured ALP activity as an early osteoblastic differentiation marker. As shown in Table 1, Tro had no significant effect on ALP activity in bone marrow cultures. Neither Pio (up to 50 μM) nor BRL (up to 50 μM) affected ALP activity (data not shown).

No Oil Red O-positive cells appeared in bone marrow cultures in the absence of TZDs. TZDs (1 μM) markedly increased the number of Oil Red O-positive cells (Table 2). 1,25-$(OH)_2D_3$ (1 nM) alone did not induce Oil Red O-positive cells or influence the effect of TZDs. As expected from previous reports (13), adipocytic differentiation was associated with increased expression of PPARγ2 and adipsin, but was

TABLE 1. Effect of troglitazone on alkaline phosphatase activity in mouse bone marrow cells

Troglitazone (μM)	ALP activity (nmol/min·μg protein)
0	33.98 ± 1.34
1	34.44 ± 1.91
3	40.08 ± 5.41
10	39.56 ± 7.54
30	33.10 ± 4.35

Mouse bone marrow cells were cultured as described in *Materials and Methods* with the indicated concentrations of troglitazone for 7 days, and ALP activity was assessed. These values are representative of three independent experiments.

TABLE 2. Effect of thiazolidinediones on oil Red-O-positive cell formation

	Oil Red-O-positive cells (cells/well)
Control	0
1,25-$(OH)_2D_3$ (1 nM)	0
Troglitazone (1 μM)	108.3 ± 6.0
Troglitazone (10 μM)	113.8 ± 9.0
Troglitazone (10 μM) + 1,25-$(OH)_2D_3$ (1 nM)	94.5 ± 11.3
BRL49653 (1 μM)	135.5 ± 7.5
BRL49653 (10 μM)	140.5 ± 18.2
BRL49653 (10 μM) + 1,25-$(OH)_2D_3$	138.5 ± 21.0

Mouse bone marrow cells were cultured as described in *Materials and Methods* with the indicated concentrations of thiazolidinediones and 1,25-$(OH)_2D_3$ for 7 days and stained with oil Red-O.

Oil Red-O-positive cells were counted. Note that no oil Red-O-positive cells were formed in the absence of thiazolidinediones. These values are representative of three independent experiments.

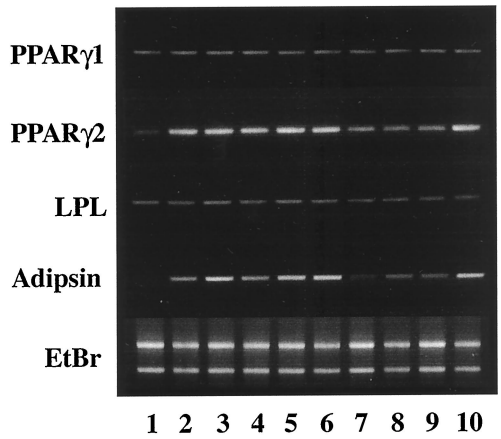

FIG. 1. Effects of TZDs and 1,25-$(OH)_2D_3$ on PPARγ1, PPARγ2, adipsin, and LPL expression. Bone marrow cells were cultured in αMEM containing 10% FBS for 7 days with the indicated test agents. Total RNA was isolated. One microgram of total RNA was electrophoresed on a 1% agarose gel, stained with ethidium bromide (EtBr), and photographed under UV transilluminator. One microgram of total RNA was reverse transcribed, and 1/20th of the reverse transcripts was amplified with PCR for PPARγ1, PPARγ2, adipsin, and LPL. PCR was performed for 25 cycles for PPARγ1 and LPL, 28 cycles for adipsin, and 30 cycles for PPARγ2. These results are representative of three independent similar experiments. Lane 1, Control; lane 2, 1 μM Tro; lane 3, 10 μM Tro; lane 4, 100 μM Tro; lane 5, 10 μM Pio; lane 6, 10 μM BRL; lane 7, 0.1 nM 1,25-$(OH)_2D_3$; lane 8, 1 nM 1,25-$(OH)_2D_3$; lane 9, 10 nM 1,25-$(OH)_2D_3$; lane 10, 10 nM 1,25-$(OH)_2D_3$ and 10 μM Tro.

not associated with the changes in PPARγ1 or LPL expression (Fig. 1).

Effects of TZDs on TRAP(+)MNC formation

Tro inhibited TRAP(+) MNC formation in bone marrow cultures induced by 1 nM 1,25-$(OH)_2D_3$ in a dose-dependent manner (Fig. 2A). At 10 μM, BRL and Pio decreased TRAP(+)MNC formation to a similar level as Tro (Fig. 2B). These TZDs also suppressed TRAP(+)MNC formation induced by 10 nM PTH (data not shown).

Effects of Tro on pit formation

We next assessed whether the inhibition by TZDs of TRAP(+)MNC formation was associated with the reduction

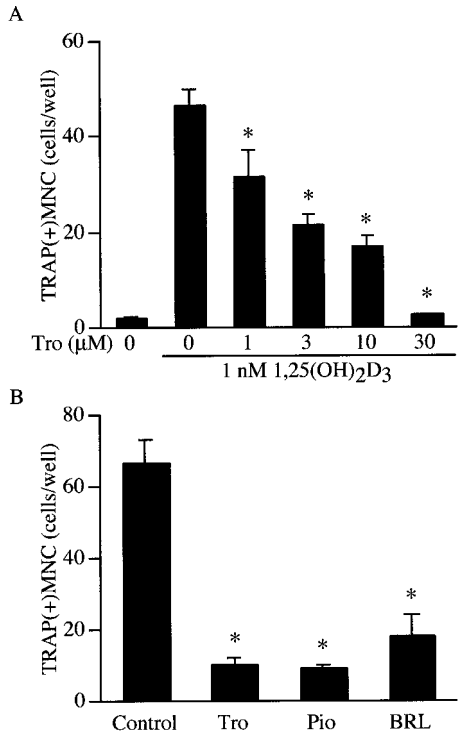

FIG. 2. A, Dose-dependent effects of Tro on TRAP(+)MNC formation. Bone marrow cells were cultured in αMEM containing 10% FBS for 7 days with or without 1 nM 1,25-$(OH)_2D_3$ and the indicated concentrations of Tro for the last 6 days. TRAP(+) cells with three or more nuclei in each well were counted. Data are expressed as the mean ± SEM (n = 4). *, Significantly different compared with the control. These results are representative of three similar experiments. B, Effects of TZDs on TRAP(+)MNC formation induced by 1,25-$(OH)_2D_3$. Bone marrow cells were cultured in αMEM containing 10% FBS for 7 days. After a day, 1 nM 1,25-$(OH)_2D_3$ was added to the culture with or without 10 μM TZDs (Tro, Pio, or BRL) and cultured for 6 days. TRAP(+) cells with three or more nuclei in each well were counted. Data are expressed as the mean ± SEM (n = 4). *, Significantly different compared with the control. These results are representative of three similar experiments.

in bone resorption using pit formation assay on dentine slices. Tro alone significantly decreased pit formation at 10 μM (Fig. 3A). Tro inhibited both 1,25-$(OH)_2D_3$- and PTH-induced pit formation at 10 μM (Fig. 3, B and C). To check whether the inhibitory effect of Tro on pit formation is mediated by general cytotoxicity or promotion of apoptosis, we assessed cell viability using the MTT assay. As shown in Table 3, Tro up to 10 μM did not significantly change MTT values, suggesting that inhibition of pit formation by Tro was not mediated by its cytotoxicity. However, at 30 μM, Tro decreased MTT values, which was less marked than its inhibition of pit formation.

Effects of IL-11 on Tro-induced changes in bone marrow cells

IL-11 is an inhibitory factor for adipocytic differentiation and an stimulatory one for osteoclast formation in bone marrow cells (20, 21). rhIL-11 as low as 0.01 nM inhibited Oil Red O-positive cell formation in the presence of 10 μM Tro (Fig. 4A). However, rhIL-11 up to 10 nM failed to restore the

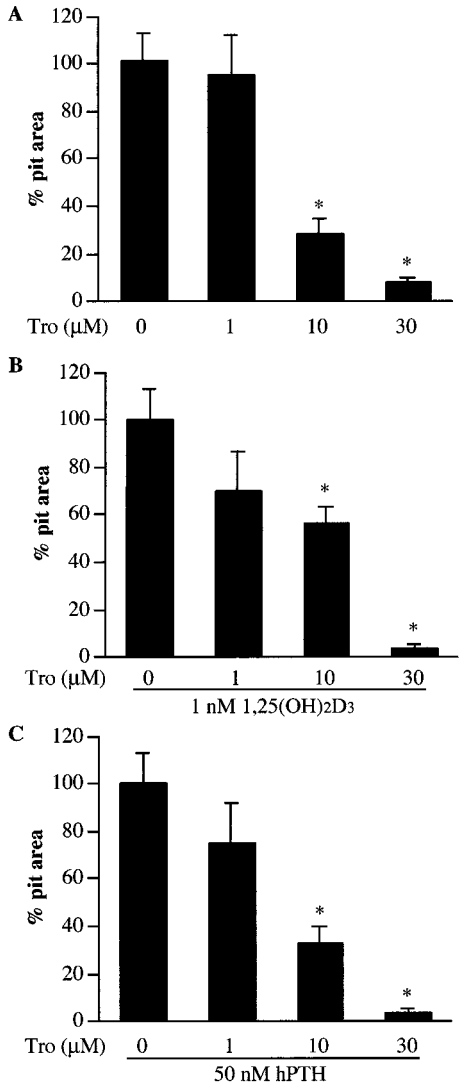

FIG. 3. Effects of Tro on pit formation. A, Bone marrow cells were cultured on a dentine slice for 4 days with Tro at the indicated doses. After cells were brushed off, dentine slices were stained with acid hematoxylin, and the total area of the pits was measured using an image analyzer. Data are expressed as the mean ± SEM (n = 9). B, Bone marrow cells were cultured on a dentine slice as described above with 1 nM 1,25-$(OH)_2D_3$ and Tro at the indicated concentrations, and pit area was measured. Data are expressed as the mean ± SEM (n = 6). C, Bone marrow cells were cultured on a dentine slice as described above with 50 nM human PTH and Tro at the indicated concentrations, and pit area was measured. Data are expressed as the mean ± SEM (n = 9). *, Significantly different from the respective control.

decreased number of TRAP(+)MNC in the presence of 10 μM Tro (Fig. 4B).

Effects of Tro on ODF and OPG expression

Recently identified ODF or OPG ligand, a membrane-bound form of cytokine expressed by bone marrow stromal cells, promotes osteoclastogenesis, whereas its decoy receptor (OPG or osteoclastogenesis inhibitory factor), also expressed by bone marrow stromal cells, inhibits osteoclastogenesis (22–25). This ODF/OPG system has been shown to play a pivotal role in the regulation of osteoclastogenesis.

TABLE 3. Effects of troglitazone on cell viability

Treatment	Troglitazone (μM)	Cell viability (% of control)
None	0	100.0 ± 1.9
	1	100.5 ± 2.9
	10	88.3 ± 4.3
	30	63.9 ± 5.2[a]
1 nM 1,25-$(OH)_2D_3$	0	100.0 ± 5.8
	1	97.8 ± 3.1
	10	85.8 ± 7.3
	30	70.4 ± 2.5[a]
1 nM PTH	0	100.0 ± 3.8
	1	103.1 ± 3.8
	10	98.5 ± 4.6
	30	63.8 ± 4.1[a]

Bone marrow cells were cultured on dentine slices for 96 h with test agents, and cell viability was assessed with MMT assay as described. Data are expressed as a percentage of the respective control values (mean ± SEM; n = 6–9).

[a] Significant difference from respective control.

To examine whether or not the inhibitory effects of TZDs on osteoclastogenesis are mediated by this cytokine pathway, we assessed Tro effects on ODF and OPG expression in our bone marrow cells with RT-PCR. As reported in other stromal cell lines (26), 1 nM 1,25-$(OH)_2D_3$ markedly increased the expression of ODF, but 10 μM Tro did not affect basal or 1,25-$(OH)_2D_3$-stimulated expression of ODF (data not shown). OPG expression was not affected by 1,25-$(OH)_2D_3$, Tro, or their combination (data not shown).

Discussion

The importance of PPARγ in the differentiation of adipocytes has been established (8). Because bone marrow stromal cells are the origin for not only adipocytes but also osteoblasts and because they are necessary for osteoclast generation, it is presumable that PPARγ activation affects differentiation of osteoblasts and/or osteoclasts. In the present study we demonstrated that TZDs inhibit the formation of osteoclast-like cells and bone resorption *in vitro*. To our surprise, although TZDs markedly promoted adipocytic differentiation of the marrow cells, this was not associated with a decrease in ALP activity, a representative early marker of osteoblastic differentiation.

Effects of TZDs on osteoblastic cells have been studied by other investigators. Gimble and co-workers (13) used a mouse bone marrow-derived adipocytic cell line, BMS2, that can be differentiated into osteoblastic cells with the stimulation by bone morphogenetic protein-2 and primary bone marrow stromal cells that are similar to the cells we used in the present study. They reported that low dose TZDs promote adipocytic differentiation, but did not touch on the possible effects of these compounds on osteoblastic differentiation. Recently, Nuttall *et al.* (27) reported that primary human osteoblast-like cells could be transdifferentiated into adipocytes after stimulation by TZD; however, whether this was associated with the loss of osteoblastic phenotype was not mentioned. The increase in adipocyte number in the bone marrow associates with the decrease in osteoblast number as evident in the aged subjects (6, 28). The reasons why the effects of TZDs on osteoblastic differentiation have not been

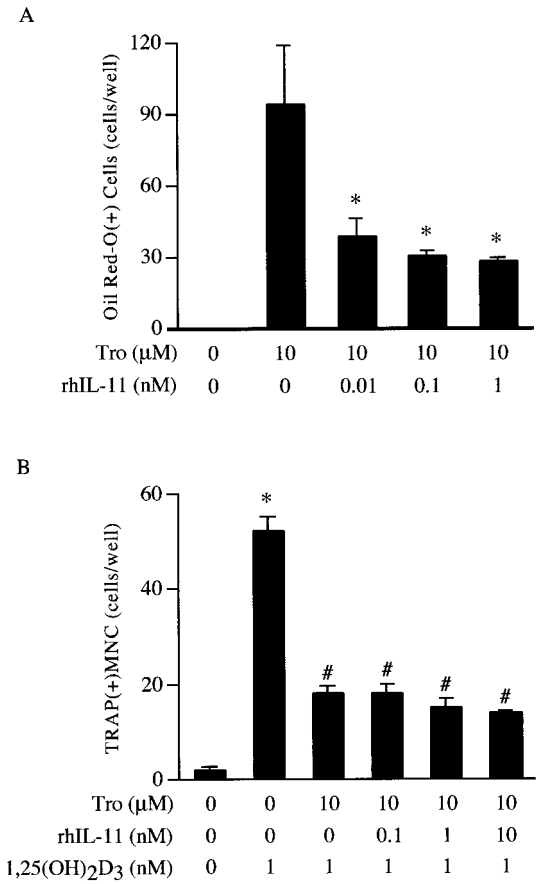

FIG. 4. Effects of interleukin-11 on Oil Red O-positive (+) and TRAP(+)MNC formation in the presence of Tro. A, Bone marrow cells were cultured in αMEM containing 10% FBS for 7 days with 10 μM Tro and the indicated doses of recombinant human interleukin-11 (rhIL-11) for the last 6 days. Oil Red O-positive cells in each well were counted. Data are expressed as the mean ± SEM (n = 4). Note that no Oil Red O-positive cells were present without Tro. *, Significantly different from the Tro alone group. B, Bone marrow cells were cultured in αMEM containing 10% FBS for 7 days with or without 1 nM 1,25-$(OH)_2D_3$, 10 μM Tro, and the indicated doses of rhIL-11 for the last 6 days. TRAP(+) cells with three or more nuclei in each well were counted. Data are expressed as the mean ± SEM (n = 4). *, Significantly different from controls; #, significantly different from the 1,25-$(OH)_2D_3$ alone group.

demonstrated *in vitro* may be due to the limitation of the presently available osteoblastic markers, such as ALP and osteocalcin, because cells in the adipocyte lineage may express them (29). As suggested by Nuttall *et al.* (27), in the bone marrow, adipocytes and osteoblasts are too closely related to be discriminated in the presence of TZDs.

In contrast to the lack of the effects on early osteoblastic differentiation, TZDs clearly inhibited osteoclast-like cell formation and bone resorption *in vitro*. Although the mechanisms by which TZDs inhibit osteoclastic bone resorption are not clear from the present study, we are able to speculate several possibilities and estimate whether they are probable based on our observations. Firstly, because both vitamin D receptor and PPARγ heterodimerize with retinoid X receptor to act as transcription factors (8), TZDs could antagonize vitamin D action at this level and inhibit osteoclastic bone resorption. However, this is unlikely, because TZDs inhibited osteoclast-like cell formation and pit formation induced not only by 1,25-$(OH)_2D_3$ but also by PTH.

Secondly, adipogenic differentiation could cause a loss of the ability of stromal cells to support osteoclastogenesis. However, the fact that IL-11 failed to restore osteoclast-like cell formation at doses that significantly blocked adipogenic differentiation of bone marrow cells suggests otherwise. Moreover, Kelly *et al.* (30) reported that a bone marrow stromal cell line, BMS-2, supports osteoclastogenesis even after adipogenic differentiation. These results suggest that adipogenic differentiation of stromal cells *per se* does not affect their capability to support osteoclastogenesis.

Thirdly, TZDs could affect actions of cytokines that control osteoclastogenesis. Osteoclastogenesis is a complex process that is under the control of a variety of hormones and cytokines (5, 31). We first examined the possible contribution of IL-11-dependent processes, because IL-11 is secreted by bone marrow stromal cells, inhibits adipogenesis, and stimulates osteoclastogenesis in the bone marrow (20, 21, 32). However, our findings suggest that the inhibitory effect of TZDs on osteoclastogenesis is not mediated by this cytokine pathway. The recently identified ODF/OPG system plays the most important role in the regulation of osteoclastogenesis dependent on stromal/hemopoietic stem cell interaction (22–25). We also explored possible involvement of this newly established pathway in TZD inhibition of osteoclastogenesis. Although our data are limited, out results suggest that the ODF/OPG pathway is unlikely to contribute to TZD effects on osteoclastogenesis. IL-6 is also secreted by bone marrow stromal cells and has similar effects as IL-11 (5, 31). IL-6 secretion by bone marrow cells was not affected by Tro up to 10 μM and was only slightly decreased at 30 μM (Takeuchi, Y., unpublished observation). Among other candidate mediators are IL-1 and tumor necrosis factor-α, both are known to promote osteoclastogenesis *in vitro* (31) and are reported to be decreased in human peripheral blood mononuclear cells with TZDs (33). These cytokines along with macrophage colony-stimulating factor are all candidates that mediate TZD's effects on bone resorption, and they are now studied in our laboratories.

Finally, TZDs could act on osteoclast progenitors instead of stromal cells. Whether osteoclast progenitors express PPARγ is unknown; however, it is expressed in several myeloid cell lines, spleen that is rich in osteoclast precursors, and tissue macrophages, which originate from the same progenitor as osteoclasts (34, 35). Interestingly, PPARγ activation in a monocytic leukemia cell line, HL-60, promotes, but does not inhibit, differentiation of these cells into mature macrophages (35). This suggests that TZDs may act on hemopoietic cells not to stimulate their osteoclastic differentiation but to push them toward macrophage differentiation. However, this possibility has to be tested in a system totally devoid of stromal cells and is beyond the scope of the present study.

Our results agree with those reported by Gimble and coworkers (13, 30, 36), in that TZDs promote adipocytic differentiation of marrow cells, but differ in several respects. First, we were unable to see any antagonistic effect of 1,25-$(OH)_2D_3$ on adipogenesis induced by TZDs. They reported that 1,25-$(OH)_2D_3$ was a potent inhibitor of adipogenesis

induced by glucocorticoid and insulin and a weak inhibitor of that induced by TZDs (36). Second, they did not see any inhibitory effect of TZDs on osteoclast-like cell formation (30). Because their culture system is completely different from ours, especially in the latter case, this could be a reason for the apparent discrepancies.

In conclusion, we demonstrated that TZDs were potent inhibitors of bone resorption *in vitro*. Our preliminary data (37) indicate that Tro administration to type 2 diabetics causes decreases in bone resorption markers before significantly improving glucose metabolism. We have previously reported that poorly controlled type 2 diabetes patients exhibit high bone resorption, which is normalized with glycemic control by treatment modalities other than TZDs (15). Taken together, our results suggest that TZDs inhibit bone resorption by dual mechanisms; locally acting on bone marrow cells and systemically improving glucose metabolism. Whether TZD treatment prevents bone loss in noninsulin-dependent diabetes patients has to be determined in future studies.

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References

1. **Beresford JN** 1989 Osteogenic stem cells and the stromal system of bone and marrow. Clin Orthop Res 240:270–280
2. **Yamaguchi A, Kahn AJ** 1991 Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tiss Int 49:221–225
3. **Asahina I, Sampath TK, Hauschka PV** 1996 Human osteogenic protein-1 induces chondroblastic, osteoblastic, and/or aipocytic differentiation of clonal murine target cells. Exp Cell Res 222:38–47
4. **Prockop DJ** 1997 Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74
5. **Suda T, Takahashi N, Martin JT** 1992 Modulation of osteoclast differentiation. Endocr Rev 13:66–80
6. **Meunier PJ, Aaron J, Edouard C, Vignon G** 1971 Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. Clin Orthop Rel Res 80:147–154
7. **Burkhardt R, Kettner G, Bohm W, Schmidmeier M, Schlag R, Frisch B, Mallmann B, Eisenmenger W, Gilg T** 1987 Changes in trabecular bone, hematopoiesis and bone marrow vessels in aplastic anemia, primary osteoporosis, and old age: a comparative histomorphometric study. Bone 8:157–164
8. **Spiegelman BM** 1998 PPAR-γ: adipogenic regulator and thiazolidinedione receptor. Diabetes 47:507–514
9. **Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM** 1995 A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptorγ and promotes adipocyte differentiation. Cell 83:813–819
10. **Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA** 1995 An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor g (PPARγ). J Biol Chem 270:12953–12956
11. **Tontonoz P, Hu E, Spiegelman BM** 1994 Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79:1147–1156
12. **Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM** 1995 15′-Deoxy-Δ12,14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 83:803–812
13. **Gimble JM, Robinson CE, Wu X, Kelly KA, Rodriguez BR, Kliewer SA, Lehmann JM, Morris DC** 1996 Peroxisome proliferator-activated receptor-γ activation by thiazolidinediones induces adipogenesis in bone marrow stromal cells. Mol Pharmacol 50:1087–1094
14. **Hu E, Tontonoz P, Spiegelman BM** 1995 Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. Proc Natl Acad Sci USA 92:9856–60
15. **Okazaki R, Totsuka Y, Hamano K, Ajima M, Miura M, Hirota Y, Hata K, Fukumoto S, Matsumoto T** 1997 Metabolic improvement of poorly-controlled non-insulin-dependent diabetes mellitus (NIDDM) decreases bone turnover. J Clin Endocrinol Metab 82:2915–2920
16. **Yamato H, Okazaki R, Ishii T, Ogata E, Sato T, Kumegawa M, Akaogi K, Taniguchi N, Matsumoto T** 1993 Effect of 24R,25-dihydroxyvitamin D_3 on the formation and function of osteoclastic cells. Calcif Tissue Int 52:255–260
17. **Takada Y, Kusuda M, Hiura K, Sato T, Mochizuki H, Nagao Y, Tomura M, Yahiro M, Hakeda Y, Kawashima H, et a** 1992 A simple method to assess osteoclast-mediated bone resorption using unfractionated bone cells. Bone Miner 17:347–359
18. **Takeuchi Y, Nakayama K, Matsumoto T** 1996 Differentiation and cell surface expression of transforming growth factor-β receptors are regulated by interaction with matrix collagen in murine osteoblastic cells. J Biol Chem 271:3938–3944
19. **Okazaki R, Durham SK, Riggs BL, Conover CA** 1995 Transforming growth factor-β and forskolin increase all classes of insulin-like growth factor-I transcripts in normal human osteoblast-like cells. Biochem Biophys Res Commun 207:963–970
20. **Girasole G, Passeri G, Jilka RL, Manolagas SC** 1994 Interleukin-11: a new cytokine critical for osteoclast development. J Clin Invest 93:1516–1524
21. **Gimble JM, Wanker F, Wang CS, Bass H, Wu X, Kelly K, Yancopoulos GD, Hill MR** 1994 Regulation of bone marrow stromal cell differentiation by cytokines whose receptors share the gp130 protein. J Cell Biochem 54:122–133
22. **Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sanders S, Van G, Tarpley J, Derby P, Lee R, Amgen EST Program, Boyle WJ** 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89:309–319
23. **Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T** 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
24. **Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K** 1998 Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. Endocrinology 139:1329–1337
25. **Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ** 1998 Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176
26. **Horwood NJ, Elliott J, Marin TJ, Gillespie MT** 1998 Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. Endocrinology 139:4743–4746
27. **Nuttall ME, Patton AJ, Livera DL, Nadeau DP, Gowen M** 1998 Human trabecular bone cells are able to express both osteoblastic and adipocytic phenotype: implication for osteopenic disorders. J Bone Miner Res 13:371–382
28. **Gimble JM, Robinson CE, Wu X, Kelly KA** 1996 The function of adipocytes in the bone marrow stroma: an update. Bone 19:421–428
29. **Dorheim MA, Sullivan M, Dandapani V, Wu X, Hudson J, Segarini PR, Rosen DM, Aulthouse AL, Gimble JM** 1993 Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J Cell Physiol 154:317–328
30. **Kelly KA, Tanaka S, Baron R, Gimble JM** 1998 Murine bone marrow stromally derived BMS2 adipocytes support differentiation and function of osteoclast-like cells in vitro. Endocrinology 139:2092–2101
31. **Mundy GR** 1996 Bone-resorbing cells. In: Favus MJ (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. Lippincott-Raven, Philadelphia, pp 16–24
32. **Kawashima I, Takiguchi Y** 1992 Interleukin-11: a novel stroma-derived cytokine. Prog Growth Factor Res 4:191–206
33. **Jiang C, Ting AT, Seed B** 1998 PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 391:82–86
34. **Greene ME, Blumberg B, McBride OW, Yi HF, Kronquist K, Kwan K, Hsieh L, Greene G, Nimer SD** 1995 Isolation of the human peroxisome proliferator activated receptor γ cDNA: expression in hematopoietic cells and chromosomal mapping. Gene Expression 4:281–299
35. **Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM** 1998 PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93:241–252
36. **Kelly KA, Gimble JM** 1998 1,25-Dihydroxyvitamin D_3 inhibits adipocyte differentiation and gene expression in murine bone marrow stromal cell clones and primary cultures. Endocrinology 139:2622–2628
37. **Okazaki R, Miura M, Hirota Y, Taguchi M, Fujita T, Tanaka K, Takeuchi Y,** Thiazolidinediones decrease bone resorption markers in patients with noninsulin dependent diabetes mellitus and inhibit osteocalst-like cell formation in vitro. 2nd Joint Meeting of the American Society for Bone and Mineral Research and the International Bone and Mineral Society, San Francisco, CA, 1998, pp S622–S623 (Abstract)