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INTRODUCTION:

Paget's disease of bone represents the second most common bone disease after osteoporosis and affects approximately 2-3 million people in the United States. We shown that bone marrow cells from patients with Paget's disease express measles virus nucleocapsid protein (MVNP) transcripts and further demonstrated that expression of the Edmonston MVNP gene in normal osteoclast (OCL) precursors results in formation of OCL that share many of the characteristics of OCL from Paget's patients. The MVNP gene contained several sense mutations, which constituted 1% of the nucleotide sequence. The pathologic significance of MVNP and associated mutations to induce abnormal OCL formation and activity in Paget's disease, is unknown (1). RANKL is a member of Tumor necrosis factor (TNF)- family member that is expressed on stromal/osteoblast cells and RANK receptor is expressed on committed osteoclast precursor cells. RANKL/RANK signaling is critical for osteoclast differentiation and bone resorption activity in vitro and in vivo (2,3). We have recently cloned and identified the Ly-6 family member, osteoclast inhibitory peptide-1 (OIP-1/hSca) which inhibits osteoclast formation and bone resorption activity. We have further demonstrated that OIP-1 significantly inhibits TNF receptor associated factor-2 (TRAF-2) and c-Jun kinase activity in osteoclast precursor cells (4). Our hypothesis is that MVNP expression in osteoclast precursors modulates the status of RANK receptor signaling molecules leading to Pagetic OCL development in Paget's disease. OIP-1 blocks these signaling events and inhibits MVNP induced osteoclastogenesis and elevated bone resorption activity in patients with Paget's disease.

BODY:

The progress on each of the proposed tasks in the statement of work is as follows:

Task (1) Determine the sensitivity of MVNP transduced osteoclast precursors to RANK Ligand (RANKL) and TNF-alpha stimulation to form pagetic osteoclasts. -- Paget's disease of bone (PD) is a chronic focal skeletal disorder characterized by excessive bone resorption followed by abundant new bone formation. Osteoclasts (OCL) from Paget's patients (PD) are abnormal and contain MVNP transcripts. We previously reported that OCL precursors from patients with Paget's disease are hyperresponsive to RANKL and 1,25-(OH)₂D₃ and that expression of MVNP in normal OCL precursors increases their responsivity to RANKL and 1,25-(OH)₂D₃. Enhanced levels of IL-6, RANKL, M-CSF and endothelin-1 have been associated with PD. However, the role of these cytokines in the progression of pagetic lesions is unclear. To further identify serum factors that are over-expressed in patients with PD, we performed 2D gel electrophoresis and mass spectrometric analysis. We identified increased serum levels of kininogen (KNG) in a patient with PD compared to normal serum. Western blot analysis of serum samples from PD patients further identified two to

five-fold increases in levels of KNG (63 kDa) compared to normal subjects. Treatment of pagetic bone marrow derived stromal/preosteoblast cells with recombinant KNG (25 ng/ml) for 24 h period resulted in a five-fold increase in the levels of phospho-HSP27 and a three-fold increase in ERK1/2 phosphorylation in these cells. However, pagetic stromal cells stimulated with KNG in the presence of ERK activation inhibitor peptide did not significantly affect the levels of phospho-HSP27. However, KNG did not affect the status of RANKL expression in these cells. KNG increased normal and pagetic marrow stromal cell proliferation at 1.4-fold and 2.5-fold respectively. KNG in the presence of an ERK inhibitor peptide did not stimulate pagetic marrow stromal cell proliferation. Furthermore, siRNA suppression of HSP27 expression significantly decreased KNG inhibition of etoposide induced caspase-3 activation and apoptosis in these cells. Therefore, KNG modulate pagetic bone marrow derived stromal/preosteoblast cell proliferation and suppress etoposide induced apoptosis through ERK and HSP27 activation respectively. These results implicate a pathophysiologic role for KNG in the pathogenesis of PD.

Task (2) Determine the RANK receptor signaling in MVNP transduced osteoclast precursors-- We have previously shown that expression of MVNP in OCL in vivo results in a bone phenotype that is characteristic of PD and support a pathophysiologic role for MVNP in PD. In collaboration with Dr. Roodman (5), we have recently identified that MVNP transduced OCL precursors demonstrate high level expression of TAF-II₁₇, a vitamin D3 receptor coactivator responsible for hyper sensitivity of these cells to vitamin D3. Data obtained in Task-1 studies indicated high levels of KNG expression in patients with PD. Therefore, studies are on going to further determine if KNG is expressed in OCL and affect RANK receptor signaling in these cells.

Task (3) Determine the effects of OIP-1 on MVNP induced RANK receptor signaling in osteoclast precursor cells-- We have made significant progress in pursuit of this aim with respect to control of OIP-1 gene expression in OCL precursor cells. To determine the molecular mechanisms that regulate OIP-1 gene expression in OCL precursor cells, we isolated and characterized the OIP-1/hSca gene (2 Kb) promoter sequence. IFN- γ (50 ng/ml) treatment of RAW 264.7 macrophage cells transfected with OIP-1 gene (-1 to -1988 bp relative to transcription start site) promoter-luciferase reporter plasmid demonstrated a significant (4 fold) increase in OIP-1 gene promoter activity. Sequence analysis of OIP-1 gene promoter region further identified a potential Stat-1 binding motif at -1629 to -1639 bp position. Stat-1 specific inhibitor, fludarabine (50 μ M) abolished IFN- γ stimulated OIP-1 gene promoter activity. Electrophoretic mobility shift assay (EMSA) further confirmed activated Stat-1 binding to the OIP-1 gene promoter sequence suggesting that IFN- γ regulates OIP-1 gene expression in OCL precursor cells through a Stat-1 dependent signaling pathway. We further show that TRADD (TNF receptor 1 associated death domain protein) negatively regulate IFN- γ signaling to enhance OIP-1 gene expression in OCL precursor cells. These results provide insights into the molecular control of OIP-

1 gene expression and inhibition of OCL activity in the bone microenvironment. Further studies are in progress to identify OIP-1 interacting proteins and to determine the role in OIP-1 inhibition of MVNP stimulated osteoclast formation.

KEY RESEARCH ACCOMPLISHMENTS:

- We have identified Kininogen (KNG) over expression in patients with Paget's disease.
- We have also shown KNG stimulates stromal/preosteoblast cell proliferation in patients with PD.
- We have further characterized IFN- γ regulation of OIP-1 gene expression and demonstrated IFN- γ induce OIP-1 expression through Stat-1 dependent signaling pathway and TRADD negatively regulate IFN- γ signaling to enhance OIP-1 gene expression in osteoclast precursor cells.

REPORTABLE OUTCOMES:

Articles:

1. Ishizuka S, Kurihara N, **Reddy SV**, Cornish J, Cundy T, Roodman GD. TEI-9647, a vitamin D receptor antagonist that inhibits osteoclast formation and bone resorption in bone marrow cultures from patients with Paget's disease. *Endocrinol.* 146:2023-30, 2005.

Abstracts:

1. Tsuruga E, Rao S, **Reddy SV**. Elevated serum high molecular weight kininogen in patients with Paget's disease of bone. 27th ASBMR meeting, 2005, Nashville, TN.
2. Srinivasan S, Tsuruga E, Ries WL, Key Jr LL, Yang S, **Reddy SV**. Interferon- γ regulation of Osteoclast Inhibitory Peptide-1 (OIP-1/hSca) gene promoter activity. 27th ASBMR meeting, 2005, Nashville, TN.
3. Yang S, Zhang YZ, **Reddy SV**, Ries W, Key LL. Osteoclasts produce interferon Ggamma: A novel autoregulation mechanism of osteoclastogenesis. 27th ASBMR meeting, 2005, Nashville TN.

CONCLUSIONS:

In conclusion, KNG modulate pagetic bone marrow derived stromal/preosteoblast cell proliferation through ERK signaling pathway and suppress etoposide induced apoptosis. Enhanced KNG levels in patients with PD further implicate a pathophysiologic role for KNG in the progression of pagetic lesions. We also demonstrate that IFN- γ regulates OIP-1 gene expression through Stat-1 dependent signaling pathway and TRADD negatively regulate IFN- γ signaling to

enhance OIP-1 gene expression in osteoclast precursor cells. Our data further suggest that OIP-1 participates in IFN- γ inhibition of osteoclast formation and bone resorption activity. Therefore, OIP-1 may have therapeutic utility to control excess bone turnover in patients with Paget's disease.

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APPENDICES:

Reprints enclosed

(23S)-25-Dehydro-1 α -Hydroxyvitamin D₃-26,23-Lactone, a Vitamin D Receptor Antagonist that Inhibits Osteoclast Formation and Bone Resorption in Bone Marrow Cultures from Patients with Paget's Disease

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Osteoclast (OCL) precursors from patients with Paget's disease (PD) and normal OCL precursors transduced with the measles virus nucleocapsid protein gene (MVNP) are hyperresponsive to 1 α ,25-dihydroxyvitamin D₃ [1 α ,25-(OH)₂D₃] and can form OCLs at physiologic concentrations of 1 α ,25-(OH)₂D₃. This hyperresponsivity to 1 α ,25-(OH)₂D₃ is due to increased expression of TATA box-associated factor II-17, a potential coactivator of the vitamin D receptor. Hyperresponsivity to 1 α ,25-(OH)₂D₃ may permit OCL formation in PD patients with low levels of 1 α ,25-(OH)₂D₃ and play a role in the pathogenesis of PD. Therefore, we tested the effects of a vitamin D antagonist, (23S)-25-dehydro-1 α -hydroxyvitamin D₃-26,23-lactone (TEI-9647), to determine its potential to inhibit the enhanced OCL formation and bone resorption seen in patients with PD. TEI-9647, by itself, was not a vitamin D receptor agonist and did not induce OCL formation *in vitro*, even at 10⁻⁶ M. However, it dose-dependently (10⁻¹⁰ M to 10⁻⁶

M) inhibited osteoclast formation induced by concentrations of 1 α ,25-(OH)₂D₃ (41 pg/ml, 10⁻¹⁰ M) detected in PD patients by bone marrow cells of patients with PD and MVNP-transduced colony-forming unit-granulocyte macrophage (CFU-GM) cells, which form pagetic-like OCL. Moreover, bone resorption by OCLs derived from MVNP-transduced CFU-GM treated with 10⁻⁹ M 1 α ,25-(OH)₂D₃ was dose-dependently inhibited by TEI-9647 (10⁻⁹ M to 10⁻⁶ M). Furthermore, 10⁻⁷ M TEI-9647 by itself did not cause 1 α ,25-(OH)₂D₃-dependent gene expression but almost completely suppressed expression of the TATA box-associated factor II-17 and 25-hydroxyvitamin D₃-24-hydroxylase genes induced by 1 α ,25-(OH)₂D₃ treatment of MVNP-transduced CFU-GM cells. These results demonstrate that TEI-9647 can suppress the excessive bone resorption and OCL formation seen in marrow cultures from patients with PD. (*Endocrinology* 146: 2023–2030, 2005)

OSTEOLASTS (OCLS) FROM patients with Paget's disease (PD) of bone are abnormal. Paget's OCLs are increased in number and size and contain many more nuclei, compared with normal OCLs. OCL precursors from PD patients are hyperresponsive to 1 α ,25-dihydroxyvitamin D₃ [1 α ,25-(OH)₂D₃] and form OCLs at low concentrations of 1 α ,25-(OH)₂D₃ (1, 2). In addition, intranuclear inclusions are present in pagetic OCLs, which are characteristic of cells chronically infected with paramyxoviruses. This observation has led to the hypothesis that PD results in part from a slow virus infection (3). In previous studies, pagetic OCLs have been shown by

immunocytochemistry to express respiratory syncytial virus and measles virus (4). Measles virus transcripts have also been detected by RT-PCR in marrow mononuclear cells in peripheral blood cells from patients with PD (5, 6).

Kurihara *et al.* (7) reported that normal OCL precursors transduced with the measles virus nucleocapsid gene formed OCLs that expressed many of the abnormal characteristics of pagetic OCLs. Moreover, they found that these cells were hyperresponsive to 1 α ,25-(OH)₂D₃. Furthermore, the measles virus nucleocapsid gene induced the hyperresponsivity of OCL precursors to 1 α ,25-(OH)₂D₃ through increased expression of TATA box associated factor II-17 (TAF_{II}-17) (8). TAF_{II}-17 is a potential coactivator of vitamin D receptor (VDR), which is also expressed in pagetic OCL precursors (8). Pagetic OCL precursors require 10–100 times less 1 α ,25-(OH)₂D₃ to form OCLs than that required for normal bone marrow cells (7, 9, 10). Furthermore, Reddy *et al.* (11) reported that measles virus infection of mouse bone marrow cells resulted in formation of pagetic-like OCLs. These results suggest that measles virus nucleocapsid protein (MVNP)-transduced normal OCL precursors are a good model of pagetic OCL precursors and that in PD, bone resorption could be enhanced by physiologic levels of 1 α ,25-(OH)₂D₃.

Recently Miura *et al.* (12) reported that a 1 α ,25-(OH)₂D₃

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Abbreviations: ALPase, Alkaline phosphatase; Ca, calcium; CFU-GM, colony-forming unit-granulocyte macrophage; FBS, fetal bovine serum; GM-CSF, granulocyte-M-CSF; M-CSF, macrophage-colony-stimulating factor; MVNP, measles virus nucleocapsid protein; OCL, osteoclast; 25-OH-D₃-24-hydroxylase, 25-hydroxyvitamin D₃-24-hydroxylase; 24R,25-(OH)₂D₃, 24R,25-dihydroxyvitamin D₃; 1 α ,25-(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; PD, Paget's disease; Pi, inorganic phosphate; RANKL, receptor activated nuclear factor- κ B ligand; TAF_{II}-17, TATA box-associated factor II-17; TEI-9647, (23S)-25-dehydro-1 α -hydroxyvitamin D₃-26,23-lactone; VDR, vitamin D receptor.

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analog, (23S)-25-dehydro-1 α -hydroxyvitamin D₃, 26,23-lactone (TEI-9647), inhibited monocyte differentiation of HL-60 cells induced by 1 α ,25-(OH)₂D₃. TEI-9647 showed significant vitamin D antagonistic activity for 25-hydroxyvitamin D₃-24-hydroxylase (25-OH-D₃-24-hydroxylase) and p21 gene expression induced by 1 α ,25-(OH)₂D₃ in HL-60 cells and human osteosarcoma cells (SaOS-2 and MG-63 cells) (12–15). Moreover, they demonstrated that TEI-9647 prevented heterodimer complex formation between the VDR and retinoid X receptor and subsequent recruitment by VDR of coactivator proteins like steroid receptor coactivator-1 (13, 15). Thus, in this model system, TEI-9647 antagonized the genomic actions of 1 α ,25-(OH)₂D₃ and interfered with VDR/VDR response element interactions with 1 α ,25-(OH)₂D₃. However, its effects on OCL formation are unknown. Because the increased osteoclastic bone resorption in PD could in part result from increased OCL formation due to the hyperresponsivity of OCL precursors to 1 α ,25-(OH)₂D₃, we tested the effects of TEI-9647 on OCL formation, bone resorption, and VDR-mediated transcriptional activity in pagetic and pagetic-like OCL precursors.

Here we report that TEI-9647 inhibited OCL formation and bone resorption by OCL formed by pagetic bone marrow cells and MVNP-transduced normal OCL precursors induced by 1 α ,25-(OH)₂D₃. TEI-9647 also suppressed TAF_{II}-17 gene expression and decreased TAF_{II}-17 protein level induced by 1 α ,25-(OH)₂D₃.

Materials and Methods

Chemicals

1 α ,25-(OH)₂D₃ and the vitamin D antagonist, TEI-9647, were synthesized in our laboratory as described previously (12). Fetal bovine serum (FBS) was purchased from Gibco-BRL (Grand Island, NY). All other chemicals and media were purchased from Sigma Chemical Corp. (St. Louis, MO), unless otherwise noted.

Subjects and cell preparation

Bone marrow cells were aspirated under 2% Xylocaine anesthesia from the iliac crest of healthy normal volunteers or three patients with PD into heparinized α MEM containing 5% FBS, as previously described (9). All PD patients had elevated alkaline phosphatase levels and had not received bisphosphonates for at least 3 months before testing. Bone marrow mononuclear cells were then isolated by separation on Hypaque-Ficoll gradients (density 1.077 g/ml), centrifuged at 400 $\times$ g for 30 min and then washed three times with α MEM, as described previously (9). The Institutional Review Board of the University of Pittsburgh approved these studies.

MVNP gene transduction of human bone marrow cells

Human bone marrow mononuclear cells were cultured for 2 d in α MEM containing 10% FBS that contained 10 ng/ml each of IL-3, IL-6, and stem cell factor (Amgen Immunex Research and Development Corp., Seattle, WA). The bone marrow cells were then cultured for an additional 48 h at 37 C in a humidified atmosphere of 5% CO₂-air at a density of 1–2 $\times$ 10⁵ cells/ml with supernatant (10% vol/vol) containing MVNP vector. Cultures were supplemented with 4 μ g/ml polybrene, 20 ng/ml IL-3, 50 ng/ml IL-6, and 100 ng/ml stem cell factor, as described previously (7). MVNP-transduced cells were suspended at 10⁶ cells/ml in α MEM containing 1.2% methylcellulose, 30% FBS, 1% deionized BSA, and 100 pg/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Amgen) with 250 μ g/ml G418. Transduced cells were plated in a volume of 1 ml in 35-mm culture dishes (Corning, New York, NY) and incubated at 37 C in a humidified atmosphere of 5% CO₂-air for 7 d, for isolation of G418 resistance colony-

forming unit-granulocyte macrophage (CFU-GM). The G418-resistant colonies were individually collected, using finally drawn pipettes, for use in OCL formation assays employing MVNP-transduced CFU-GM cells.

OCL formation induced by 1,25-(OH)₂D₃

Normal bone marrow mononuclear cells (10⁶ cells/ml) were dispersed into α MEM containing 20% horse serum and were seeded in 96-well multiplates (Becton Dickinson Labware, Franklin Lakes, NJ) at 100 μ l/well. 1 α ,25-(OH)₂D₃ (10⁻⁸ M), 10⁻¹¹ M to 10⁻⁶ M TEI-9647, alone or in combination, was each added into a well. Half of the media was replaced two times a week, and the cultures were continued for 3 wk at 37 C in an incubator of 5% CO₂-air. The OCLs that formed were then fixed with 2% formaldehyde and tested for cross-reactivity with the monoclonal antibody 23C6, which recognizes the OCL vitronectin receptor (generously provided by Michael Horton, Rayne Institute, Bone and Mineral Center, London, UK), using a Vectastain-ABC-AP kit (Vector Laboratories, Burlingame, CA), as described previously (9). Cells that cross-reacted with the 23C6 antibody and had three or more nuclei were scored as OCLs using an inverted microscope. In the case of MVNP-transduced CFU-GM-derived cells or pagetic bone marrow cells, 10⁻¹⁰ M 1 α ,25-(OH)₂D₃ was used instead of 10⁻⁸ M 1 α ,25-(OH)₂D₃, which was used for normal bone marrow cells.

Osteoclastic bone resorption on dentin slices

MVNP-transduced CFU-GM cells (2 $\times$ 10⁶ cells/ml), as a model for pagetic OCL precursors, or normal marrow cells were dispersed into α MEM media containing 20% horse serum and seeded on mammoth dentin slices (Wako Pure Chemical Industries Ltd., Osaka, Japan) in 96-well multiplates at 100 μ l/well. Receptor activator of nuclear factor- κ B ligand (RANKL; 50 ng/ml) and macrophage-CSF (M-CSF; 50 ng/ml; Wako) were added into each well to induce OCL formation. Half of the media containing 20% horse serum, RANKL, and M-CSF was replaced two times a week, and the cultures were continued for 3 wk at 37 C in an atmosphere of 5% CO₂-air. After 3 wk, the cells were incubated for 3 d in media lacking RANKL and M-CSF. Then the cultures were treated with 10⁻⁹ M or 10⁻⁸ M 1 α ,25-(OH)₂D₃, 10⁻⁹ M to 10⁻⁶ M TEI-9647, or a combination of both for 10 d. After 10 d, OCLs on the dentin slices were removed by overnight incubation in 0.25% trypsin at 37 C, and the resorption lacunae were stained with hematoxylin. Pit area was quantified by image analysis.

Gene expression induced by 1 α ,25-(OH)₂D₃ in MVNP-transduced bone marrow cells

MVNP-transduced CFU-GM cells (5 $\times$ 10⁵ cells/ml) were cultured in α MEM containing 10% FBS with 10⁻¹⁰ M to 10⁻⁸ M 1 α ,25-(OH)₂D₃, 10⁻¹⁰ M to 10⁻⁷ M TEI-9647, or a combination of 10⁻⁸ M 1 α ,25-(OH)₂D₃ and 10⁻¹⁰ M to 10⁻⁷ M TEI-9647 for 12 h. Total RNA extraction and RT-PCR were carried out as described previously (7). The gene-specific primers for TAF_{II}-17 (GenBank accession no. U57693) were 5'-CATGCCATGGCTATGAACCAGTTTGGCCCTCA-3' (sense) and 5'-ATACTGCAGTATTCTTGGTTGTTTCCG-3' (antisense). The gene-specific primers for 25-OH-D₃-24-hydroxylase (GenBank accession no. L13286) were 5'-ATTACCTGAGAATCAGAGGCCACG-3' (sense) and 5'-GCCAAATGCAGTTTAAGCTCTGCT-3' (antisense). The conditions for amplification were as follows: a 5-min initiation step at 94 C; 35 cycles at 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min; and finally an extension step at 72 C for 7 min. PCR products were separated by 2% agarose gel electrophoresis and were revealed with ethidium bromide staining under UV light. The relative amounts of TAF_{II}-17 mRNA and 25-OH-D₃-24-hydroxylase mRNA were determined by densitometry and compared with β -actin mRNA expression levels. The PCRs were performed during the linear phase of the reaction.

Western blot analysis of TAF_{II}-17

To evaluate TAF_{II}-17 protein levels, MVNP-transduced CFU-GM cells were cultured with 10⁻⁹ M 1 α ,25-(OH)₂D₃, 10⁻⁷ M TEI-9647, or a combination of 10⁻⁹ M 1 α ,25-(OH)₂D₃ and 10⁻¹⁰ M to 10⁻⁷ M TEI-9647 for 4 d, and then cell lysates were prepared as described previously (8).

The protein concentration of the lysates was determined by the Bradford method, and the same amount of protein from each lysate was loaded onto SDS-PAGE 12% polyacrylamide gel. The blotted peptides on the gel were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% skim milk in Tris-HCl-buffered saline containing 0.1% Tween 20, the membranes were incubated with antirabbit-TAF_{II}-17 antibody (generously provided by Dr. R. G. Roeder, Rockefeller University, New York, NY) at 1:2000 dilution in Tris-HCl-buffered saline containing 0.1% Tween 20 and 1% BSA for 1 h. The blot was incubated for 1 h with horseradish peroxidase-conjugated goat antirabbit IgG (Dako, Carpinteria, CA), and the bands were visualized with an enhanced chemiluminescence system (Amersham Life Science, Arlington Heights, IL).

Modified mammalian two-hybrid assays

The TAF_{II}-17 cDNA was digested with *Sma*I and *Sal*I and inserted into the pM vector (pM-TAF_{II}-17) (CLONTECH Laboratories Inc., Palo Alto, CA). The full-length cDNA for human VDR was released by *Eco*RI digestion from the pSG5 vector and fused in-frame into the pVP16 vector, which contains the activation domain of a herpesvirus (CLONTECH) (pVP16-hVDR) (13). To examine the interaction of TAF_{II}-17 and VDR, 0.5 μ g of pM-TAF_{II}-17, 0.5 μ g of pVP16-hVDR, and 0.5 μ g of pGVP2-GAL4BS together with 0.25 μ g of plasmid containing β -galactosidase cDNA were transfected into NIH3T3 cells using Lipofectamine (Gibco). Twenty-four hours later, vehicle, 10^{-8} M $1\alpha,25$ -(OH)₂D₃, 10^{-7} M TEI-9647, and a combination of 10^{-8} M $1\alpha,25$ -(OH)₂D₃ and 10^{-7} M TEI-9647 were added. After 24 h of incubation, the luciferase activity of the cell lysates was examined and standardized using β -galactosidase activity.

Measurement of serum concentrations of vitamin D metabolites calcium, inorganic phosphate and alkaline phosphatase

Serum samples were collected from nine patients with PD and 10 age-matched healthy normal volunteers (3–5 ml/subject). The serum concentrations of vitamin D metabolites were measured as described previously (16). Total serum calcium (Ca), inorganic phosphate (Pi), and alkaline phosphatase (ALPase) were determined using commercially available kits (calcium C test, inorganic phosphorus P test, and ALPase B test, Wako).

Statistical analysis

Significance was evaluated using a two-sided, unpaired Student's *t* test, with *P* < 0.01 considered significant.

Results

Serum concentrations of vitamin D metabolites in patients with PD

Our hypothesis is that increased OCL formation and bone resorption in PD is due in part to the hyperresponsivity of pagetic OCL precursors to $1\alpha,25$ -(OH)₂D₃. This hyperresponsivity to $1,25$ -(OH)₂D₃ results in OCL formation at physiologic levels of $1\alpha,25$ -(OH)₂D₃, something that occurs at very low levels in normals. However, measurements of $1\alpha,25$ -(OH)₂D₃ levels in PD patients have only rarely been reported and are conflicting (17, 18). Therefore, we determined the concentrations of vitamin D metabolites, Ca, and Pi in the serum of nine patients with PD and 10 age-matched normal healthy volunteers. The concentrations of vitamin D metabolites, 25-OH-D, 24,25-(OH)₂D, and $1\alpha,25$ -(OH)₂D, Ca, and Pi in the serum of patients with PD were very similar to their concentrations in the serum of age-matched normal healthy volunteers. No abnormality of vitamin D metabolism was detected in patients with PD (Table 1). In particular, the concentrations of $1\alpha,25$ -(OH)₂D₃ in the serum of patients with PD were 41.0 ± 9.1 pg/ml serum (10^{-10} M) and were the same as that of age-matched normal

TABLE 1. Serum concentrations of vitamin D metabolites, calcium, and inorganic phosphate in patients with Paget's disease and age-matched normal volunteers

	Patients with Paget's disease (n = 9)	Normal volunteers (n = 10)
25-OH-D	40.5 $\pm$ 11.1	39.9 $\pm$ 9.5
24,25-(OH) ₂ D	2.6 $\pm$ 1.6	2.4 $\pm$ 1.1
$1\alpha,25$ -(OH) ₂ D	41.0 $\pm$ 9.1	38.8 $\pm$ 12.1
Calcium (mg/dl)	9.8 $\pm$ 0.5	9.7 $\pm$ 0.3
Inorganic phosphate (mg/dl)	3.4 $\pm$ 0.4	3.4 $\pm$ 0.5

Vitamin D metabolites, calcium, and inorganic phosphate in the serum were measured as described in *Materials and Methods*. Data are shown as the mean $\pm$ SD.

healthy volunteers. ALPase activity in the serum of patients with PD was significantly higher than that of age-matched normal healthy volunteers (data not shown).

Effects of TEI-9647 on OCL formation

We then confirmed our previous results that these levels of $1\alpha,25$ -(OH)₂D₃ could induce OCL formation in marrow cultures from PD patients and MVNP-transduced normal OCL precursors. Using bone marrow cells from patients with PD or MVNP-transduced CFU-GM, 10^{-11} M to 10^{-9} M $1\alpha,25$ -(OH)₂D₃ dose-dependently stimulated OCL formation. OCL formation in these cultures was significantly increased above control levels at 10^{-11} M $1\alpha,25$ -(OH)₂D₃ and reached maximum levels at 10^{-9} M $1\alpha,25$ -(OH)₂D₃. In contrast, although $1\alpha,25$ -(OH)₂D₃ 10^{-10} M to 10^{-7} M dose-dependently stimulated OCL formation in normal marrow cultures, it was not significantly increased until 10^{-9} M $1\alpha,25$ -(OH)₂D₃ was added to the cultures and reached maximum levels at 10^{-7} M $1\alpha,25$ -(OH)₂D₃ (Fig. 1, A–C).

We then tested the effects of TEI-9647 on OCL formation induced by $1\alpha,25$ -(OH)₂D₃ in bone marrow cell cultures from normal volunteers, patients with PD, or MVNP-transduced CFU-GM. TEI-9647 did not stimulate OCL formation in any of the cultures, even at high concentrations (10^{-6} M). In contrast, TEI-9647 dose-dependently blocked OCL formation induced by 10^{-8} M $1\alpha,25$ -(OH)₂D₃ in normal marrow cultures, which was significant at 10^{-9} M TEI-9647 or higher. TEI-9647 also dose-dependently blocked OCL formation induced by 10^{-10} M $1\alpha,25$ -(OH)₂D₃ in pagetic marrow cultures. TEI-9647 (10^{-10} M) significantly inhibited OCL formation induced by 10^{-10} M $1\alpha,25$ -(OH)₂D₃. TEI-9647 (10^{-6} M) completely blocked OCL formation (Fig. 1B). TEI-9647 also inhibited OCL formation by MVNP-transduced CFU-GM cells in a similar fashion as pagetic marrow cultures (Fig. 1C).

Effects of TEI-9647 on osteoclastic bone resorption

To determine whether TEI-9647 inhibits VDR-mediated osteoclastic bone resorption by pagetic-like OCLs, we examined the effects of TEI-9647 on bone resorption by OCLs that were formed on dentin slices by MVNP-transduced CFU-GM cells treated with RANKL and M-CSF and then activated by 10^{-9} M $1\alpha,25$ -(OH)₂D₃. Large OCLs were present with 10^{-9} M $1,25$ -(OH)₂D₃ treatment, compared with vehicle-treated cells (data not shown). Treatment with TEI-9647 did not change the number of OCLs on the dentin, compared with treatment with 10^{-9} M $1\alpha,25$ -(OH)₂D₃ (Fig. 2). Pit for-

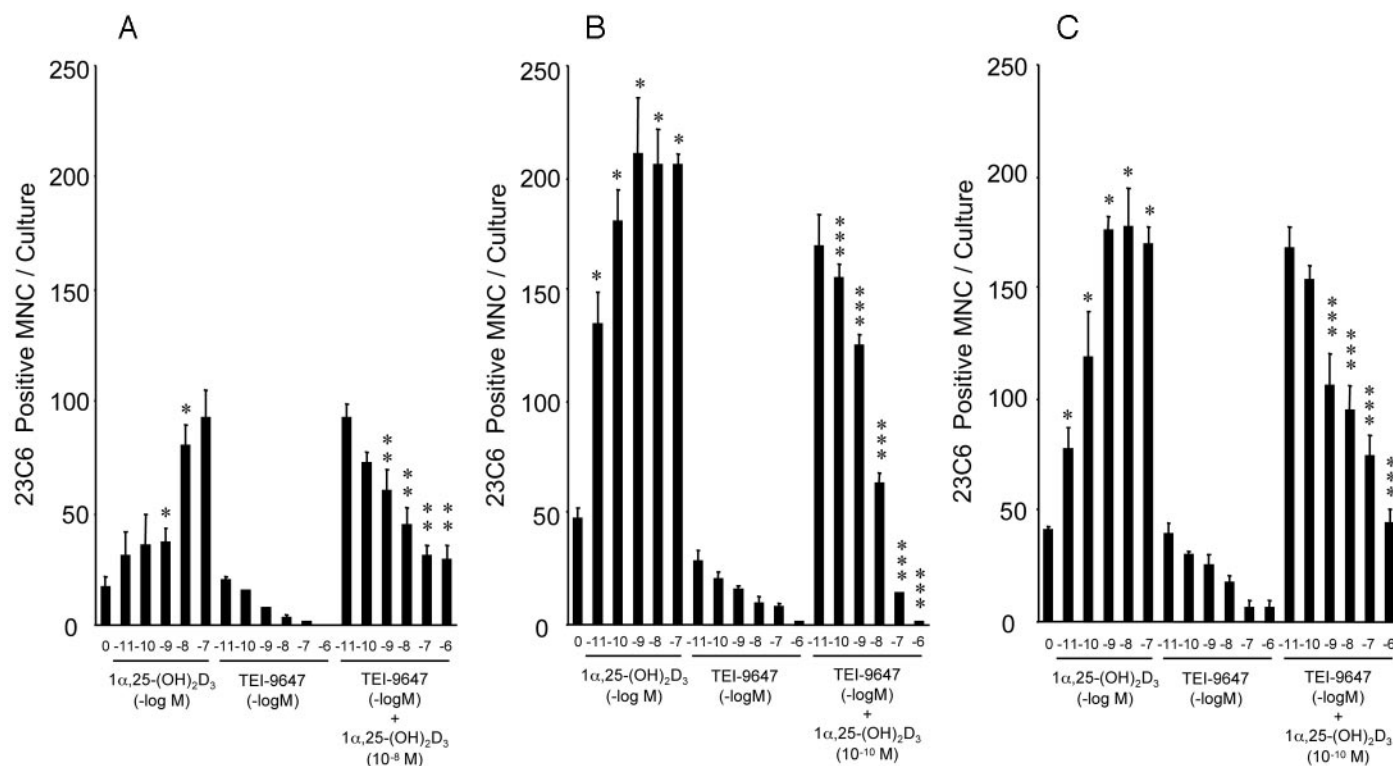


FIG. 1. Effect of TEI-9647 on OCL formation by normal bone marrow cells treated with $1\alpha,25-(OH)_2D_3$ (A), patients with PD (B), and MVNP-transduced CFU-GM cells (C). Bone marrow mononuclear cells were dispersed into α MEM containing 20% horse serum, and 10^5 cells were seeded in 96-well plates. The cultures were continued for 3 wk with vitamin D_3 analogs (*i.e.* 10^{-11} M to 10^{-7} M $1\alpha,25-(OH)_2D_3$, 10^{-11} M to 10^{-6} M TEI-9647, or a combination of 10^{-11} M to 10^{-6} M TEI-9647 and $1\alpha,25-(OH)_2D_3$ (10^{-8} M in normal cells, 10^{-10} M in PD and MVNP-transduced CFU-GM cells). The media were replaced two times a week. Multinucleated cells that cross-reacted with the 23C6 antibody and had three or more nuclei were scored as OCLs. Data are expressed as the mean $\pm$ SD ($n = 4$). *, Significantly different from control cultures (media alone), $P < 0.01$; **, significantly different from cultures treated with 10^{-8} M $1\alpha,25-(OH)_2D_3$, $P < 0.01$ (A); ***, significantly different from cultures treated with 10^{-10} M $1\alpha,25-(OH)_2D_3$ treatment, $P < 0.01$ (B and C). MNC, Multinuclear cells.

mation activated by 10^{-9} M $1\alpha,25-(OH)_2D_3$ is shown in Fig. 2B. $1\alpha,25-(OH)_2D_3$ (10^{-9} M) markedly enhanced osteoclastic bone resorption. TEI-9647 (10^{-9} M to 10^{-6} M) dose-dependently blocked osteoclastic bone resorption induced by 10^{-9} M $1\alpha,25-(OH)_2D_3$, and 10^{-7} M TEI-9647 almost completely blocked bone resorption. The area resorbed by the OCLs was quantified by image analysis and is shown in Fig. 2C.

Effect of TEI-9647 on TAF_{II-17} and 25-OH- D_3 -24-hydroxylase gene expression and TAF_{II-17} protein levels

We then performed time-course and dose-response experiments to assess the antagonistic actions of TEI-9647 on VDR-mediated gene transcription using MVNP-transduced CFU-GM cells as a model for pagetic OCL precursors. TEI-9647 (10^{-7} M) did not induce expression of the TAF_{II-17} and 25-OH- D_3 -24-hydroxylase genes but almost completely suppressed their expression induced by exposure to 10^{-9} M $1\alpha,25-(OH)_2D_3$ for 6 and 12 h. TAF_{II-17} and 25-OH- D_3 -24-hydroxylase gene expression reappeared by 24 h after treatment with TEI-9647 (data not shown), demonstrating that TEI-9647 was not toxic to the cells. In dose-response experiments, at 12 h after treatment with $1\alpha,25-(OH)_2D_3$ and TEI-9647, 10^{-10} M to 10^{-8} M $1\alpha,25-(OH)_2D_3$ but not TEI-9647 dose-dependently increased TAF_{II-17} and 25-OH- D_3 -24-hydroxylase gene expression. As previously reported, TAF_{II-17}

was expressed in the absence of added $1\alpha,25-(OH)_2D_3$ in MVNP-transduced CFU-GM cells, but 25-OH- D_3 -24-hydroxylase was not (Fig. 3A). TEI-9647 dose-dependently suppressed TAF_{II-17} and 25-OH- D_3 -24-hydroxylase gene expression induced by 10^{-9} M $1\alpha,25-(OH)_2D_3$, and 10^{-8} M TEI-9647 completely suppressed TAF_{II-17} and 25-OH- D_3 -24-hydroxylase gene expression. The relative effects of TEI-9647 on TAF_{II-17} and 25-OH- D_3 -24-hydroxylase mRNA expression in three independent experiments are shown in Fig. 3B.

To determine whether TEI-9647 decreased protein levels of TAF_{II-17} induced by $1\alpha,25-(OH)_2D_3$, we measured the relative levels of TAF_{II-17} in MVNP-transduced CFU-GM cells by Western blot analysis as shown in Fig. 3C. MVNP-transduced CFU-GM cells produced a 17- and 34-kDa protein, which reacted with TAF_{II-17} antibody in the absence of $1\alpha,25-(OH)_2D_3$. The 34-kDa band is most likely a dimer of TAF_{II-17} . Low concentration of $1\alpha,25-(OH)_2D_3$ (10^{-9} M) increased the levels of TAF_{II-17} . In contrast, 10^{-10} M to 10^{-7} M TEI-9647 dose-dependently decreased the levels of TAF_{II-17} enhanced by 10^{-9} M $1\alpha,25-(OH)_2D_3$.

Effect of TEI-9647 on the interaction between VDR and TAF_{II-17}

To confirm the role of TAF_{II-17} in VDR-mediated gene transcription, we investigated the interaction of VDR with

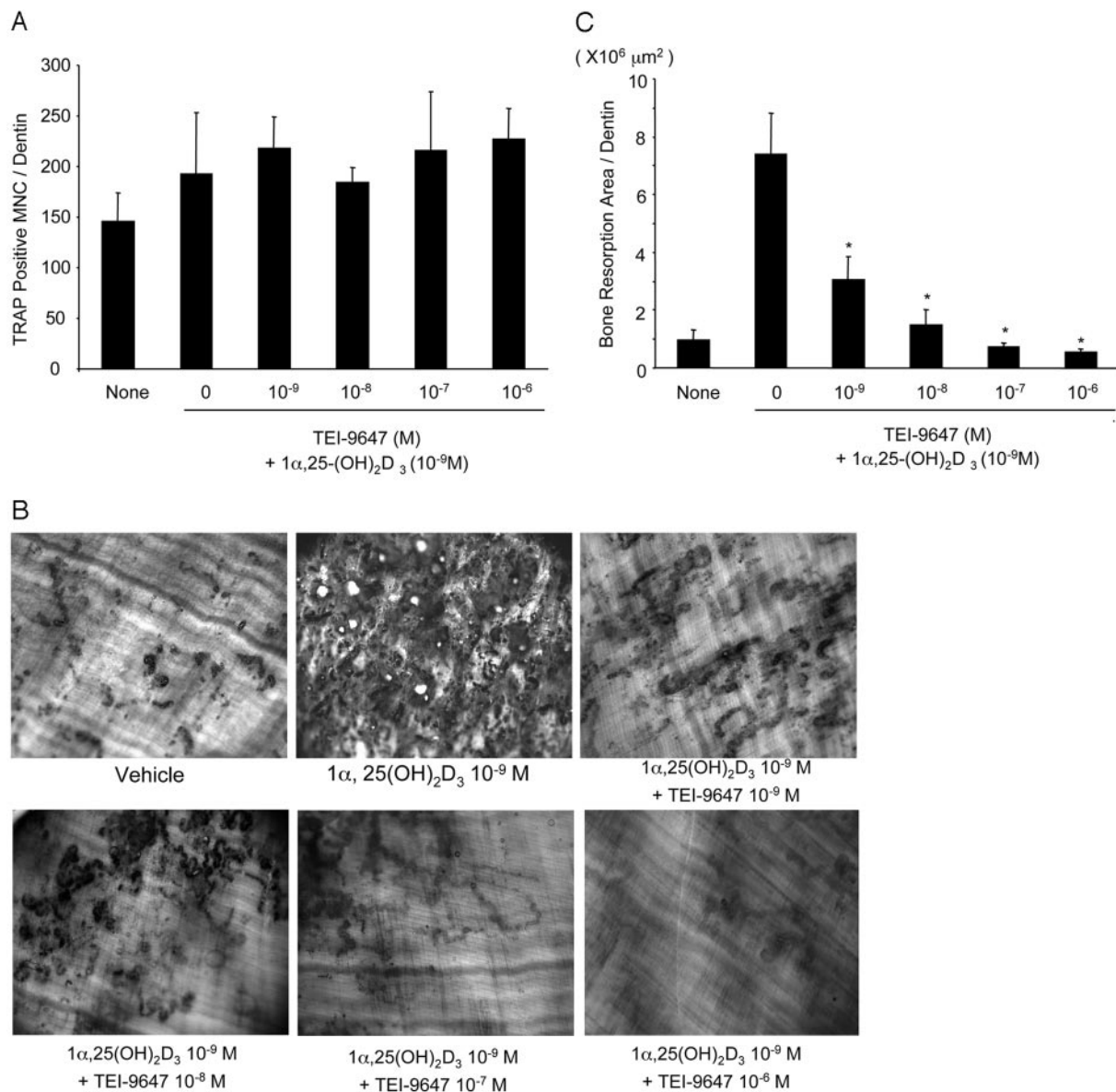


FIG. 2. Effects of TEI-9647 on bone resorption induced by $1\alpha, 25(\text{OH})_2\text{D}_3$. MVNP-transduced CFU-GM cells were dispersed into α MEM containing 20% horse serum, and 10^5 cells were seeded in 96-well plate. RANKL (50 ng/ml) and M-CSF (50 ng/ml) were added into a well as stimulators of OCL formation. The cultures were continued for 3 wk. The media containing 20% horse serum, RANKL, and M-CSF were replaced two times per week. After 3 wk, media lacking RANKL and M-CSF were added and the cells incubated for 3 d. The OCLs that formed were then activated by the treatment with 10^{-9} M $1\alpha, 25(\text{OH})_2\text{D}_3$, 10^{-9} M to 10^{-6} M TEI-9647, or a combination of both for 10 d. After 10 d, OCLs on the dentin slices were stained for tartrate-resistant acid phosphatase (TRAP). A, Number of OCLs present on dentin slices. Data are expressed as the mean $\pm$ SD ($n = 4$). *, Significantly different from control cultures (media alone), $P < 0.01$. The OCLs were released by incubation with trypsin overnight, and the resorption lacunae were stained with hematoxylin. B, Pits on dentin slice. C, Pit area. Data are expressed as the mean $\pm$ SD ($n = 4$). *, Significantly different from control cultures (media alone), $P < 0.01$. MNC, Multinuclear cells.

TAF_{II}-17. Protein-protein interaction between VDR and TAF_{II}-17 was examined using the mammalian two-hybrid system. An expression vector in which human TAF_{II}-17 was fused to GAL4DBD (pM-TAF_{II}-17) was used as the bait construct, and the human VDR (pVP16-hVDR) was used as the prey vector (Fig. 4A). VDR-mediated gene transcription was induced when 10^{-8} M $1\alpha, 25(\text{OH})_2\text{D}_3$ was added with the VDR and TAF_{II}-17 constructs, but it was significantly suppressed by treatment with 10^{-7} M TEI-9647 (Fig. 4B).

Discussion

We and others previously reported that OCL activity is markedly increased in patients with PD and that OCL formation is abnormal (9, 19). OCL precursors from patients with PD have increased sensitivity to $1, 25(\text{OH})_2\text{D}_3$ and can form OCLs at physiologic levels of $1, 25(\text{OH})_2\text{D}_3$, something that normal OCL precursors cannot do. As shown in Table 1, patients with PD have levels of $1, 25(\text{OH})_2\text{D}_3$ in their serum

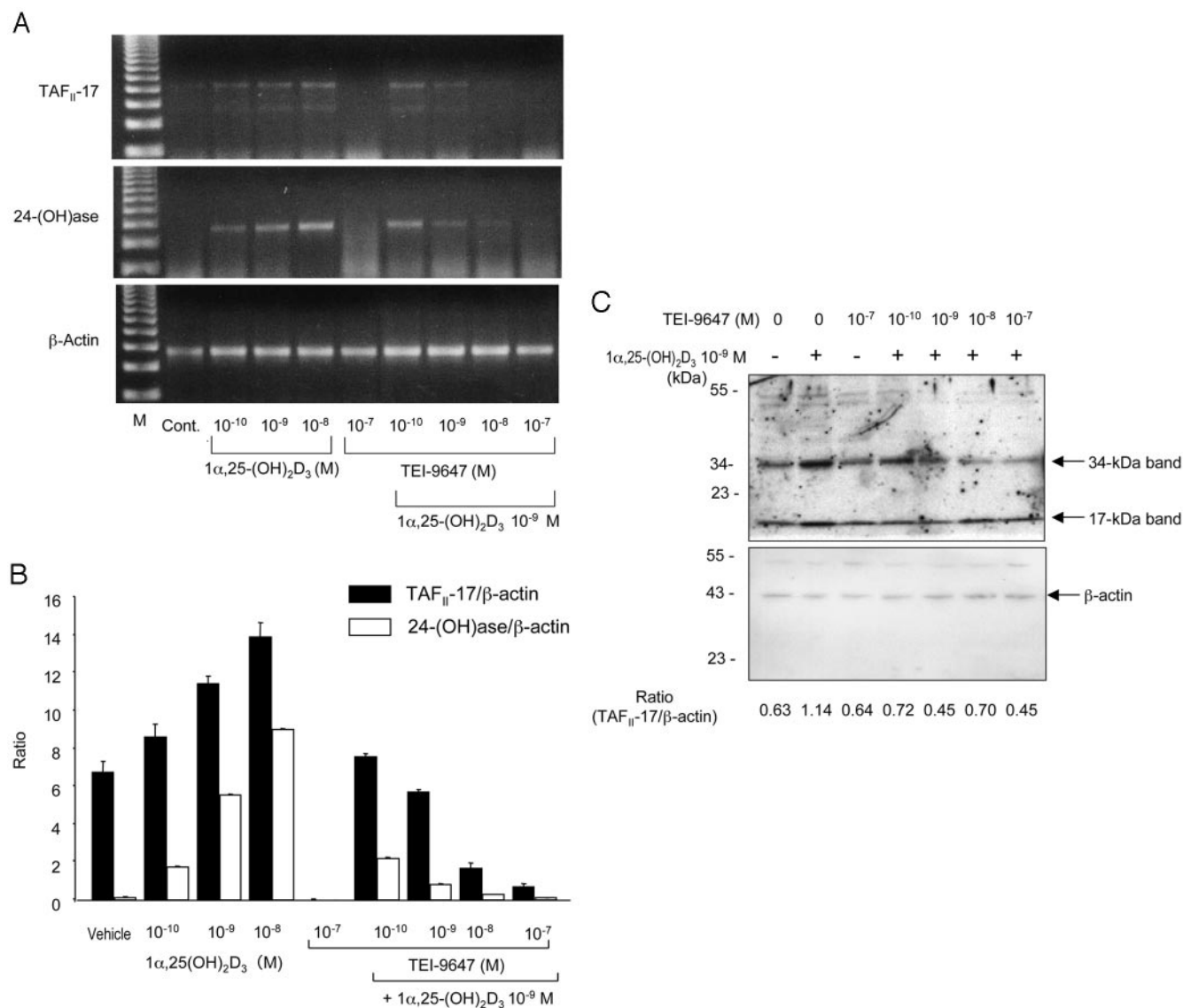


FIG. 3. Effect of TEI-9647 on TAF_{II}-17 gene expression and TAF_{II}-17 protein levels. A, Effect of TEI-9647 on TAF_{II}-17 and 25-OH-D₃-24-hydroxylase gene expression induced by 1α,25-(OH)₂D₃ in MVNP-transduced CFU-GM-cells. MVNP-transduced CFU-GM cells were cultured in αMEM containing 10% FBS for 12 h with 10⁻¹⁰ M to 10⁻⁸ M 1α,25-(OH)₂D₃, 10⁻⁷ M TEI-9647, or a combination of 10⁻⁹ M 1α,25-(OH)₂D₃ and 10⁻¹⁰ M to 10⁻⁷ M TEI-9647. Total RNA was extracted and RT-PCR was carried out. PCR products were separated by 2% agarose gel electrophoresis and were revealed with ethidium bromide staining as described in *Materials and Methods*. Cont, Control (nontreated, media alone). B, Densitometric analysis of three independent PCR experiments examining the effects of TEI-9647 on TAF_{II}-17 and 25-OH-D₃-24-hydroxylase mRNA levels. C, Western blot analysis of TAF_{II}-17 protein. To evaluate TAF_{II}-17 protein levels, MVNP-transduced CFU-GM cells were cultured with 1α,25-(OH)₂D₃, TEI-9647, or a combination of 1α,25-(OH)₂D₃ and TEI-9647 for 4 d, and then cell lysates were prepared. Western blot analysis of TAF_{II}-17 protein in the cell lysates was carried out as described in *Materials and Methods*. Seventeen- and 34-kDa bands both reacted with the anti-TAF_{II}-17. The 34-kDa is a dimer of TAF_{II}-17. D, Densitometric analysis of three independent Western blot analyses for the effects of TEI-9647 on TAF_{II}-17 expression.

that do not differ from normals and are sufficient to induce osteoclastogenesis. These findings agree with previous work by Devlin *et al.* (17) and Foldes *et al.* (18), who showed similar levels of 1,25-(OH)₂D₃ in PD patients and normals. In addition, as shown in Fig. 2, C and D, these levels of 1,25-(OH)₂D₃ can enhance the bone resorption capacity of preformed OCLs that are similar to pagetic OCLs.

Therefore, we tested TEI-9647, a vitamin D analog that can block the effects of 1,25-(OH)₂D₃ on hematopoietic cell differentiation, for its capacity to block OCL formation *in vitro*

by marrow samples from patients with PD and MVNP-transduced normal OCL precursors, as a surrogate model of pagetic OCL precursors. We previously reported that MVNP-transduced osteoclast precursors are very similar to pagetic osteoclast precursors (7). As shown in Figure 1, 1,25-(OH)₂D₃ (10⁻¹¹ M) induced high levels of OCL formation in PD cultures but minimally affected OCL formation in normal marrow cultures. TEI-9647 dose-dependently inhibited osteoclast formation in both normal and pagetic marrow cultures. OCL formation in PD cultures was more sensitive

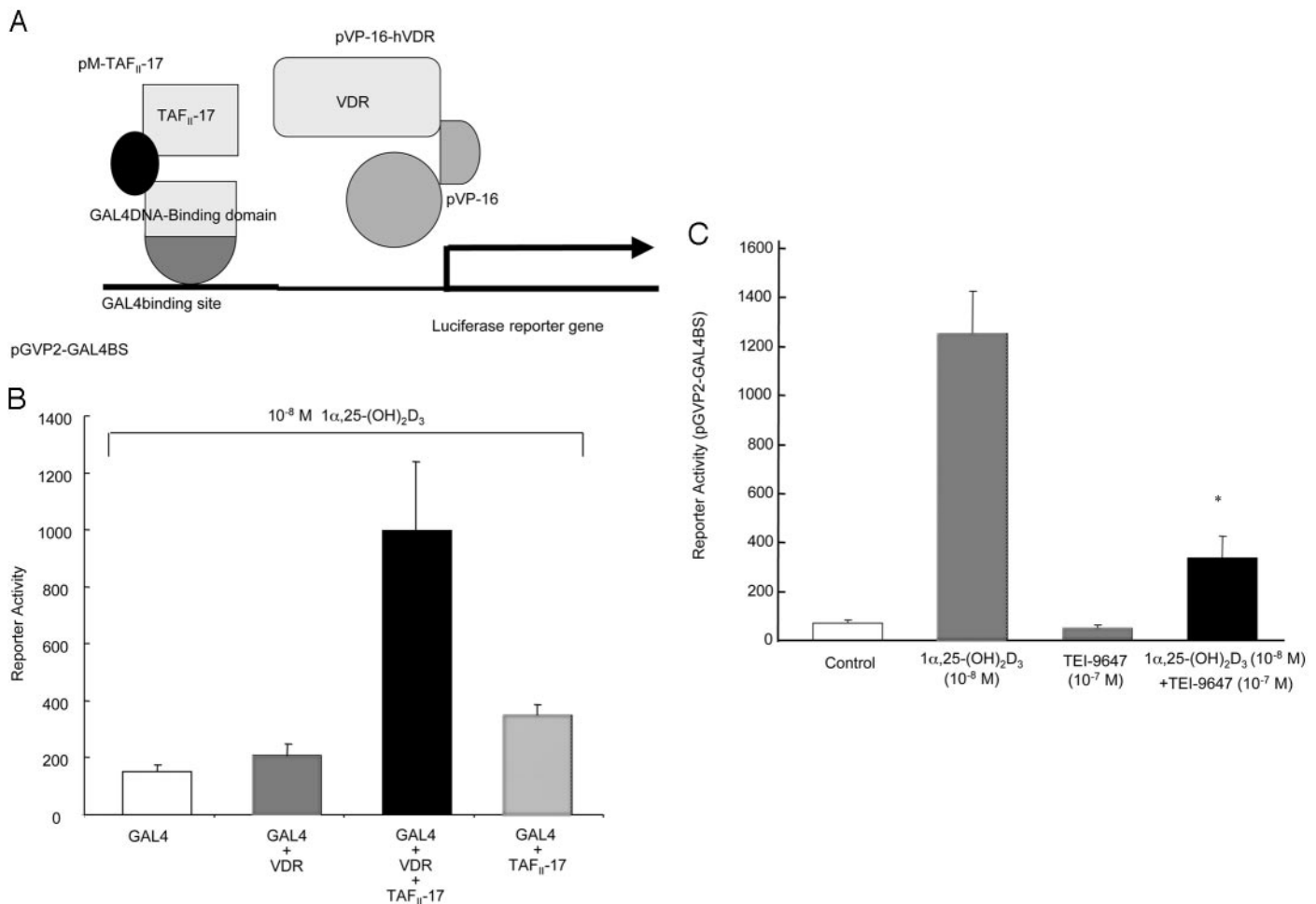


FIG. 4. Effect of TEI-9647 on the interaction between VDR and TAF_{II}-17. **A**, Modified mammalian two-hybrid assay was used to examine the interaction between VDR and TAF_{II}-17. The TAF_{II}-17 cDNA was inserted at the *EcoRI* site of the pM vector, which contains a GAL4 DNA-binding domain (pM-TAF_{II}-17). **B**, The interaction of VDR and TAF_{II}-17 was examined by measuring the reporter activity in NIH3T3 cells transduced with pM-TAF_{II}-17, pVP16-hVDR, and the reporter plasmid containing the GAL4 DNA-binding sites (pGVP2-GAL4BS). GAL4 protein is a transcriptional activator that binds to DNA. 1,25-(OH)₂D₃ (10⁻⁸ M), TEI-9647 (10⁻⁷ M), or both were added after transfection. After 24 h of incubation, the luciferase activity of the cell lysates was determined as described in *Materials and Methods*. Results are expressed as the mean $\pm$ SEM. *, $P < 0.01$, compared with cells treated with 10⁻⁸ M 1,25-(OH)₂D₃. Similar results were obtained in three independent experiments. No reporter activity was seen in control incubations lacking TAF_{II}-17 or VDR constructs.

to TEI-9647. TEI-9647 (10⁻¹¹ to 10⁻⁹ M) decreased OCL formation approximately 3-fold in PD cultures, whereas it decreased normal OCL formation only approximately 2-fold. Of interest, TEI-9647 also decreased basal OCL formation in both normal and pagetic marrow cultures. This decrease in basal OCL formation was not due to toxicity of TEI-9647 for hematopoietic cells. We have shown in preliminary studies that TEI-9647 does not block the growth of hematopoietic precursor cells such as CFU-GM and in the current study reversibly inhibited VDR-mediated gene transcription (Fig. 3A). Furthermore, TEI-9647 was not toxic to preformed OCL because OCL numbers were unchanged when OCLs were treated with TEI-9647 (Fig. 2B). Both 1,25-(OH)₂D₃-stimulated and basal OCL formation were not significantly inhibited in normal marrow cultures until the TEI-9647 concentration was 10⁻⁹ M, whereas pagetic marrow cultures were significantly inhibited by 10⁻¹⁰ M TEI-9647. TEI-9647 (10⁻⁸ M) decreased OCL formation almost to basal levels in marrow cultures from patients with PD who were treated with phys-

ologic levels of 1,25-(OH)₂D₃ (10⁻¹⁰ M) (Fig. 1B). These data demonstrate that TEI-9647 inhibited OCL formation induced by 1,25-(OH)₂D₃ and that basal osteoclastogenesis in marrow cultures may in part be mediated by the low levels of 1,25-(OH)₂D₃ present in the horse serum in these cultures.

TEI-9647 also blocked VDR-mediated gene transcription. As shown in Fig. 3A, TEI-9647 markedly decreased TAF_{II}-17 expression induced by 1,25-(OH)₂D₃ both at the mRNA and protein level. Furthermore, mammalian two hybrid assays demonstrated that TEI-9647 blocked the interaction between TAF_{II}-17 and VDR. These results suggest that TEI-9647 or similar agents might in addition to decreasing OCL formation and activity could also affect the sensitivity of these precursors to 1,25-(OH)₂D₃. Kurihara *et al.* (8) reported that increased expression of TAF_{II}-17 is in part responsible for the hypersensitivity of pagetic OCL precursors to 1,25-(OH)₂D₃. They showed that transfecting TAF_{II}-17 into normal OCL precursors results in increased sensitivity of these precursors to 1,25-(OH)₂D₃. Furthermore, an antisense to TAF_{II}-17

blocked OCL formation by pagetic osteoclast precursors induced by low levels of $1,25\text{-(OH)}_2\text{D}_3$. TEI-9647 suppresses expression of TAF_{II}-17 in MVNP-transduced normal OCL precursors, which have many of the characteristics of pagetic OCL, including hypersensitivity to $1,25\text{-(OH)}_2\text{D}_3$ and high levels of TAF_{II}-17. These results suggest that TEI-9647 may be a drug that can both block the enhanced OCL formation and bone resorption in patients with PD in response to $1,25\text{-(OH)}_2\text{D}_3$ and possibly change the sensitivity of pagetic OCL precursors to $1,25\text{-(OH)}_2\text{D}_3$.

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Elevated Serum High Molecular Weight Kininogen in Patients with Paget's Disease of Bone

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Presentation Number: SA467

Paget's disease of bone (PD) is a chronic focal skeletal disorder characterized by excessive bone resorption followed by abundant new bone formation, with eventual replacement of normal bone marrow by vascular and fibrous tissue. Enhanced levels of IL-6, M-CSF and endothelin-1 have been associated with PD, implicated in its pathogenesis and indicator of disease activity. RANK ligand (RANKL), a critical osteoclastogenic factor that is expressed on marrow stromal/osteoblast cells is upregulated in areas involved with PD. We have recently demonstrated that heat shock factor-2 (HSF-2) is a downstream target of fibroblast growth factor-2 (FGF-2) to induce RANKL expression in stromal/preosteoblast cells. These data suggest that heat shock proteins/heat shock factors may play an important role in bone remodeling. To further identify serum factors that are over-expressed in patients with PD, we performed 2D gel electrophoresis and mass spectrometric analysis of serum from patients. We identified increased serum levels of high molecular weight glycosylated kininogen (KNG) in a patient with PD compared to normal serum. Western blot analysis of serum samples (6 μ g total protein) from three PD patients further identified two to five-fold increase in levels of KNG heavy chain (63 kDa) compared to five normal subjects. However, there was no significant change in the serum levels KNG light chain (58 kDa) expression in these patients. We then examined the effects of KNG on human bone marrow derived stromal cells (SAKA-T cells). Treatment of SAKA-T cells with recombinant KNG (25 ng/ml) for 24 hr period resulted in a five-fold increase in the levels of phospho-HSP-27 and a three-fold increase in ERK1/2 phosphorylation in these cells. However, SAKA-T cells stimulated with KNG in the presence of ERK activation inhibitor peptide-1 (25 μ M) which binds to ERK2 and prevents interaction with MEK did not significantly affect the levels of phospho-HSP-27. These data suggest that KNG may play an important role in modulating stromal/preosteoblastic cell proliferation/differentiation. These results may implicate a potential pathophysiologic role for KNG in the progression of pagetic lesions in patients with PD.

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Osteoclasts Produce Interferon Ggamma: A Novel Autoregulation Mechanism of Osteoclastogenesis.

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Presentation Number: SU279

Osteoclasts are multinucleated cells formed from hemopoietic cells, and their formation is tightly controlled by a network of cytokines produced in the bone microenvironment. While there are intensive efforts in recent years to understand the mechanisms of osteoclastogenesis by positive stimulators, very little attention has been given to negative regulatory mechanisms.

Interferon gamma (IFN- γ), a systemic immune factor produced primarily by T and NK cells, inhibits osteoclastogenesis and bone resorption. In this study, we demonstrated that osteoclasts express IFN- γ by immunostaining and RT-PCR analysis. ELISA analysis showed that IFN- γ produced by osteoclasts reached 0.37 ng/ml, which is above the minimum dose (0.2 ng/ml) required to inhibit osteoclastogenesis. IFN- γ mediates its biological effects through the receptor. The presence of IFN- γ receptors in osteoclasts is demonstrated by their binding activity to ^{125}I -labeled IFN- γ . Scatchard plot analysis revealed that osteoclast progenitors have an average of 5300 IFN- γ receptor sites/cell. We have also identified that IFN- γ inhibits osteoclast precursor proliferation significantly. These data suggest that a negative feedback loop exists by which osteoclasts negatively control their own differentiation and formation by producing IFN- γ .

Evidence that supports our study is obtained from the IFN- γ receptor null mice, which have a more rapid onset of arthritis and bone resorption, as more osteoclasts appeared in the IFN- γ receptor (-/-) mice than in wild type mice. IFN- γ failed to suppress osteoclastogenesis in bone marrow cell cultures derived from the IFN- γ receptor (-/-) mice. These data suggest that osteoclast formation in the IFN- γ receptor (-/-) mice lacks negative regulation when the IFN- γ signaling is interrupted. Without such negative control, more osteoclasts are formed and lead to excess bone resorption in the IFN- γ receptor (-/-) mice.

Osteoclast-derived IFN- γ may be one of the major negative regulators of osteoclastogenesis in the bone microenvironment. This regulation may function more efficiently than the systemic source of IFN- γ , through paracrine and autocrine mechanisms. This negative regulation limits the number of

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Interferon- γ Regulation of Osteoclast Inhibitory Peptide-1 (OIP-1/hSca) Gene Promoter Activity

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Osteoclast formation and activity are regulated by autocrine/paracrine factors produced in the bone microenvironment. We have previously identified and characterized osteoclast inhibitory peptide-1 (OIP-1/hSca), a member of Ly-6 gene family. Immune cell products such as IFN- γ are potent inhibitors of osteoclast formation. We have previously shown that IFN- γ stimulates OIP-1/hSca expression in osteoclast precursor cells. However, IFN- γ modulates gene expression through complex regulatory mechanisms. To determine the molecular mechanisms that regulate OIP-1 gene expression in osteoclast precursor cells, we isolated and characterized the human OIP-1/hSca gene (2 Kb) promoter. IFN- γ treatment of RAW 264.7 cells transfected with an OIP-1 gene promoter-luciferase reporter plasmid demonstrated a significant (4 fold) enhancement of luciferase activity. Analysis of the OIP-1 gene promoter sequence identified a potential Stat-1 binding motif at -1625 to -1640 bp position relative to the transcription initiation site. We therefore examined Stat-1 regulation of OIP-1 gene promoter activity in response to IFN- γ treatment to osteoclast precursor cells. IFN- γ stimulation of RAW 264.7 cells transfected with OIP-1 gene (-1 to -1988 bp) promoter-luciferase reporter construct in the presence of Stat-1 inhibitor, fludarabine (50 μ M) abolished IFN- γ stimulated OIP-1 gene promoter activity. Electrophoretic mobility shift assay (EMSA) demonstrated activated Stat-1 binding to the OIP-1 gene promoter sequence. Antiphospho-Stat-1 antibody addition to the EMSA reaction abolished Stat-1 binding to the OIP-1 gene promoter region. Furthermore, nuclear extracts derived from fludarabine treated IFN- γ stimulated RAW 264.7 cells did not demonstrate binding to the Stat-1 consensus sequence present in the OIP-1 gene promoter. However, IFN- γ treatment did not stimulate the activity of a OIP-1 gene promoter deletion construct that lacked the Stat-1 binding region. These data suggest that IFN- γ regulates OIP-1 gene promoter activity in osteoclast precursor cells through a Stat-1 dependent signaling pathway.