Dexamethasone Inhibits the Formation of Multinucleated Osteoclasts *via* Down-regulation of β_3 Integrin Expression

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Although glucocorticoids are known to affect osteoclast differentiation and function, there have been conflicting reports about the effect of glucocorticoids on osteoclast formation, leading to the assumption that microenvironment and cell type influence their action. We explored the effect of the synthetic glucocorticoid analog dexamethasone on the formation of osteoclasts. Dexamethasone inhibited the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts without affecting the formation of TRAP-positive mononuclear cells in a coculture of mouse osteoblasts and bone marrow cells. Dexamethasone did not inhibit mRNA expression levels of the receptor activator of nuclear factor-kB ligand and osteoprotegerin, the essential regulators of osteoclastogenesis. Dexamethasone down-regulated the expression of β_3 integrin mRNA and protein but did not alter expression of other osteoclast differentiation marker genes. Both dexamethasone and echistatin, a β_3 integrin function blocker, inhibited TRAP-positive multinucleated osteoclast formation but not TRAP-positive mononuclear cell formation. These results suggest that dexamethasone inhibits the formation of multinucleated osteoclasts, at least in part, through the down-regulation of β_3 integrin, which plays an important role in the formation of multinucleated osteoclasts.

Key words: Dexamethasone, Osteoclast, Vitronectin receptor, β_3 Integrin, Fusion

INTRODUCTION

The skeleton undergoes continuous remodeling throughout life, and the maintenance of normal bone mass depends on a balance between bone resorption and subsequent bone formation (Roodman, 1996). Osteoblasts synthesize and deposit bone matrix to increase bone mass. Osteoclasts, large multinucleated phagocytes, are fully differentiated cells primarily responsible for bone resorption. There is evidence that osteoclast differentiation depends on cell-to-cell contact between hematopoietic osteoclast precursors and osteoblasts. These close interactions are regulated by the receptor activator nuclear factor-κB ligand (RANKL)/osteoprotegerin (OPG)/RANK and macrophage colony-stimulating factor/c-Fms systems (reviewed in Duong and Rodan, 2001).

Osteoporosis is a well-known side effect of long-term

glucocorticoid therapy (Canalis, 1996), mainly due to a decrease in osteoblastic bone formation. Glucocorticoids also have direct effects on bone resorption, although there have been conflicting reports about their effect on osteoclast formation and activation. It has been shown that dexamethasone (DEX), a synthetic glucocorticoid analog, inhibits formation of osteoclast-like cells in bone-marrow cultures (Pharoah and Heersche, 1986). Glucocorticoids also increase calcitonin receptor (CTR) expression and sensitivity to calcitonin in human osteoclasts (Wada et al., 2001). However, many in vitro experiments have demonstrated that glucocorticoids increase osteoclast formation and activity both directly and indirectly (Kaji et al., 1997; Hofbauer et al., 1999; Richards et al., 2000; Takuma et al., 2003). Thus, glucocorticoids seem to have biphasic action on osteoclast formation depending on the cellular microenvironment and cell type on which they exert their effect.

Osteoclasts express high levels of $\alpha_v\beta_3$ integrin, also called the vitronectin receptor (VNR). Adhesion of osteoclasts to the bone surface involves the interaction of integrins with extracellular matrix proteins within the bone

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matrix (Duong *et al.*, 2000). Several lines of evidences have suggested a role of VNR in osteoclast function. The knockout or mutation of the β_3 subunit of VNR impairs bone resorptive activity of osteoclasts but not osteoclast formation itself (McHuge *et al.*, 2000; Feng *et al.*, 2001). However, VNR does seem to play a role in the formation of multinucleated osteoclasts by affecting cell migration (Nakamura *et al.*, 1998).

In this study, we explored the effects of DEX on the formation of osteoclasts, particularly the fusion of osteoclast precursors to produce multinucleated osteoclasts. We found that DEX inhibited the formation of multinucleated osteoclasts, at least in part, through the down-regulation of β_3 integrin.

MATERIALS AND METHODS

Materials

ICR mice were purchased from the Daehan Experimental Animal Center (Umsung-Kun, Korea). α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), trypsin-EDTA, penicillin and streptomycin, the SUPER-SCRIPT First-Strand Synthesis System, and other cultural reagents were obtained from Invitrogen (Carlsbad, CA, U.S.A.). $[\alpha\text{-}^{32}P]dCTP$ and the Megaprime DNA labeling system were obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.). ExpressHyb Hybridization Solution was purchased from Clontech (Palo Alto, CA, U.S.A.) and 1,25-$(OH)_2$vitaminD$_3$ was obtained from Calbiochem-Novobiochem Corp. (San Diego, CA, U.S.A.). Antibodies to β_3 integrin and α_v integrin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated secondary antibodies and Super-signal chemiluminescent substrate were purchased from Pierce (Rockford, IL, U.S.A.). PE-conjugated anti-mouse CD14 antibody was purchased from PharMingen (San Diego, CA, U.S.A.). Normal donkey serum was obtained from Immunoresearch (West Groove, PA, U.S.A.). Easy-BLUE reagent was purchased from iNtRON Biotechnology (Seoul, Korea). Primers for reverse transcription-polymerase chain reaction (RT-PCR) and TaKaRa Taq polymerase were purchased from TaKaRa (Otsu, Japan). Reagents for tartrate-resistant acid phosphatase (TRAP) staining, DEX, ponceau S, and echistatin were obtained from Sigma (St. Louis, MO, U.S.A.).

Coculture of osteoblastic cells and bone marrow cells

Mouse calvarial osteoblastic cells and bone marrow cells were obtained as previously described (Kim *et al.*, 2002). Osteoblastic cells and bone marrow cells were plated together at a density of 7.5×10^3 and 1.5×10^5 cells/ well, respectively, in 48-well culture plates and cultured for

7 days in a-MEM supplemented with 10% FBS. During the culture period, 10^{-7} M DEX or 10^{-7} M echistatin was added to the coculture during the first 4 days, the last 3 days, or the entire culture period in the presence of 10^{-8} M 1,25-$(OH)_2$vitaminD$_3$. At the end of 7 days, the cells were subjected to TRAP staining using a leukocyte acid phosphatase assay kit following the manufacturer's protocol. The number of TRAP(+) multinucleated cells containing three or more nuclei were counted under a light microscope. Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals at Seoul National University.

Analysis of osteoclast differentiation markers, RANKL, and OPG expression

Osteoblastic cells and bone marrow cells were cocultured in α-MEM supplemented with 10% FBS with or without 10^{-7} M DEX in the presence of 10^{-8} M 1,25-$(OH)_2$vitaminD$_3$. After 2, 3, 4, 5, and 6 days of culture, total cellular RNA was isolated using easy-BLUE reagent and used for cDNA synthesis. cDNA was synthesized from 1 μg of total RNA with Superscript RT and PCR was performed using 10% of the cDNA produced. Amplified osteoclast markers were CTR, VNR (β_3 subunit), matrix metalloproteinase-9 (MMP-9), cathepsin K (CTSK), carbonic anhydrase 2 (CA2), RANK, and TRAP. PCR products were electrophoresed on a 1.2% agarose gel and visualized under UV light after ethidium bromide staining. The following mouse genes and their primer sequences were used for PCR: CTR-forward (f) 5'-TGAAAAGGCGGA-ATCT-3', CTR-reverse (r) 5'-AGGAACATGTGCTTGTG-3'; VNR-f 5'-GCTCAGATGAGACTTTG-3', VNR-r 5'-ATC-AACAATGAGCTGGA-3'; MMP-9-f 5'-CGAGACATGATC-GATGA-3', MMP-9-r 5'-CGAGACATGATCGATGA-3'; CTSK-f 5'-TCAAGGTTCTGCTGCTA-3', CTSK-r 5'-GAG-CCAAGAGAGCATAT-3'; CA2-f 5'-CACCCTTCCAAG-ATCTTATA-3', CA2-r 5'-ATCCATTGTGTTGTGGTATG-3'; RANK-f 5'-AAGATGGGTCCAGAAGACGGT-3', RANK-r 5'-CATAGAGTCAGTTCTGCTCGGA-3'; TRAP-f 5'-TGA-CAAGAGGTTCCAGGA-3', TRAP-r 5'-AGCCAGGAC-AGCTGAGTG-3'; OPG-f 5'-TGAGTGTGAGGAAGGGCG-TTAC-3', OPG-r 5'-TTCCTCGTTCTCTCAATCTC-3'; RANKL-f 5'-ATCAGAAGACAGCACTCACT-3', RANKL-r 5'-ATCTAGGACATCCATGCTAATGTTC-3'; β-actin-f 5'-GGACTCCTATGGTGGGTGACGAGG-3', β-actin-r 5'-GGGAGAGCATAGCCCTCGTAGAT-3'.

RT-PCR result was confirmed with northern blot analysis. Twenty-five μg of total RNA were separated on a 1.2% agarose/formaldehyde gel and then transferred to a nylon membrane by capillary blotting. The mouse β_3 integrin and β-actin cDNA probes were produced by PCR and subsequently by random primer labeling with $[\alpha\text{-}^{32}P]dCTP$ using the Megaprime DNA labeling system.

Hybridization and all of the washing steps were carried out according to the ExpressHyb Hybridization Solution instruction manual. The membrane was exposed to a phosphor-image plate and the hybridized bands were identified using a Bas 1500 phosphorimager (Fuji Photo Film Co. Ltd., Tokyo, Japan).

To identify protein expression levels, osteoblastic cells and bone marrow cells were cultured together under the same conditions as above and used for western blot analysis. Cells were lysed with SDS lysis buffer (62.5 mM Tris-HCl; pH 6.8, 2% w/v sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol). Thirty μg of each protein sample was subjected to sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (8% gel). Immunoblotting was initiated by incubating the blot for 60 min at room temperature with blocking buffer (0.1% Tween-20, 5% w/v nonfat dry milk in phosphate-buffered saline). Blots were probed with anti-β_3 integrin or anti-α_v integrin antibodies. After the blots were incubated with horseradish peroxidase-conjugated secondary antibody, immunoreactivity was detected by Supersignal chemiluminescent substrate using a Las plus image analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Immunofluorescent staining

Osteoblastic cells and bone marrow cells at a density of 1.5×10^4 and 3.0×10^5 cells per well, respectively, were cultured in 4-well chamber slides for 4 days in α-MEM supplemented with 10% FBS in the presence of 10^{-8} M 1,25-$(OH)_2$vitaminD$_3$. After 4 days of culture, cells were fixed with 3.7% paraformaldehyde and incubated with blocking buffer (5% normal donkey serum in 1× phosphate buffered saline). After incubation with goat anti-β_3 antibody, cells were incubated with FITC-conjugated donkey anti-goat IgG antibody and PE-conjugated anti-mouse CD14 antibody, and then mounted. The cells were viewed on a LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, U.S.A.).

Statistical analysis

All results are presented as mean ± S.E. Statistical significance was analyzed by Student's t-test. P values less than 0.01 were considered to indicate a statistically significant difference.

RESULTS

DEX inhibited the formation of multinucleated osteoclasts

To clarify the modulating effect of DEX on osteoclast formation, we cocultured mouse bone marrow cells with osteoblastic cells in the presence of 1,25-$(OH)_2$vitaminD$_3$. The effect of DEX on osteoclast formation was evaluated

in the presence of osteoblastic cells because osteoblasts co-exist in the bone microenvironment, and mediate factor-stimulated osteoclast formation through the regulation of RANKL and/or OPG expression (Suda *et al.*, 1999; Takahashi *et al.*, 1999). The coculture system used in this study resulted in a pattern of osteoclast differentiation similar to that seen in earlier studies (reviewed in Duong and Rodan, 2001). At the beginning of coculture, bone marrow cells that adhered onto the osteoblastic cells proliferated for 2 to 3 days and TRAP(+) mononuclear cells appeared afterward. On the 5th day of coculture, TRAP(+) multinucleated cells began to appear and increase in number during the remaining culture periods (data not shown). This result demonstrates that the fusion of mononuclear precursors into multinucleated osteoclasts starts around the 4th or 5th day of coculture.

DEX significantly inhibited the formation of TRAP(+) multinucleated osteoclasts in a dose-dependent manner (Fig. 1A). As shown in Fig. 1B, the majority of TRAP(+) cells in this condition were mononuclear (Fig. 1B). Treatment with DEX for only the first 4 days had an inhibitory effect similar to DEX treatment for the entire culture period (Fig.
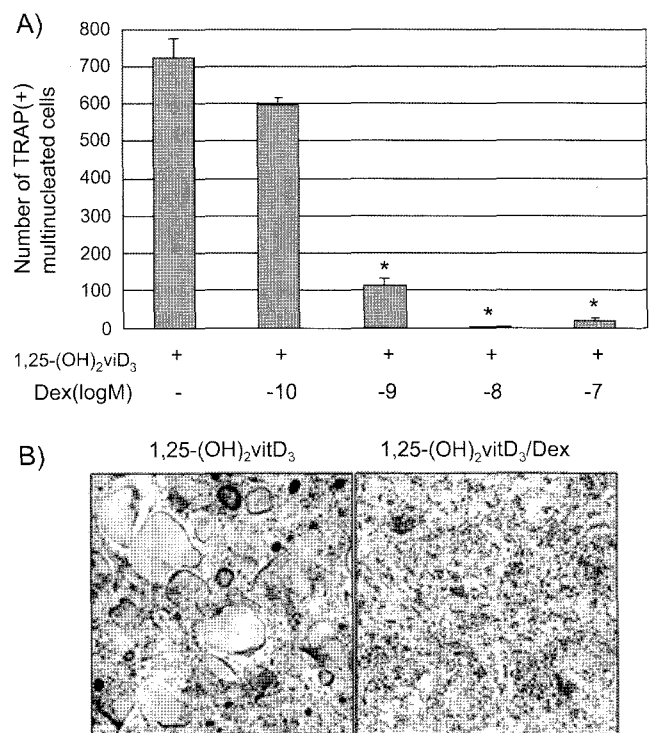

Fig. 1. Effect of dexamethasone on the multinucleated osteoclast formation. Mouse osteoblastic cells and bone marrow cells were cocultured for 7 days with or without dexamethasone in the presence of 10^{-8} M 1, 25-$(OH)_2$vitamin D$_3$. TRAP staining was carried out and number of TRAP (+) cells containing 3 or more nuclei were counted as multinucleated cells. A) Data represent means ± S.E. (N=6). Dex, dexamethasone, * p<0.01, compared to control (1,25-$(OH)_2$vitaminD$_3$ alone). B) Original magnification ×40.

2). These results suggest that DEX treatment during early osteoclast differentiation influences the fusion of mononuclear precursors, but not the formation of TRAP(+) prefusion osteoclasts.

Neither RANKL nor OPG mediated the inhibitory effect of DEX

RANKL on the surface of osteoblasts/stromal cells interacts with RANK on the hemopoietic cell surface, resulting in osteoclast differentiation. OPG is also secreted

by osteoblasts/stromal cells and inhibits osteoclast differentiation by interrupting the RANKL-RANK interaction (reviewed in Duong and Rodan, 2001). Thus, the regulation of the relative expression of RANKL and OPG in osteoblasts/stromal cells is critical to the modulation of osteoclastogenesis. We examined the expression of RANKL and OPG in coculture to isolate the mechanism by which DEX inhibits osteoclast fusion. It has been reported that DEX further enhances $1,25\text{-}(OH)_2vitaminD_3$-induced RANKL expression in ST2 cells when added simultaneously (Yasuda *et al.*, 1998). However, as shown in Fig. 3, the addition of DEX did not significantly affect the expression levels of RANKL and OPG in our cocultures, suggesting that RANKL or OPG may not be the factors that mediate the inhibition of multinucleated osteoclast formation by DEX.
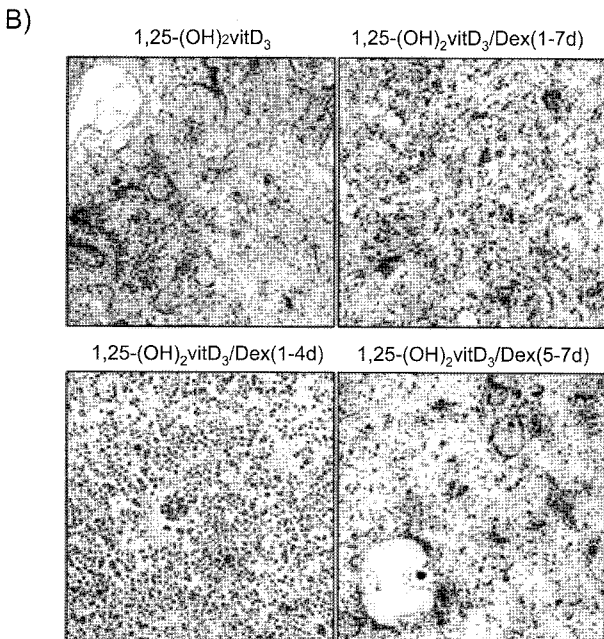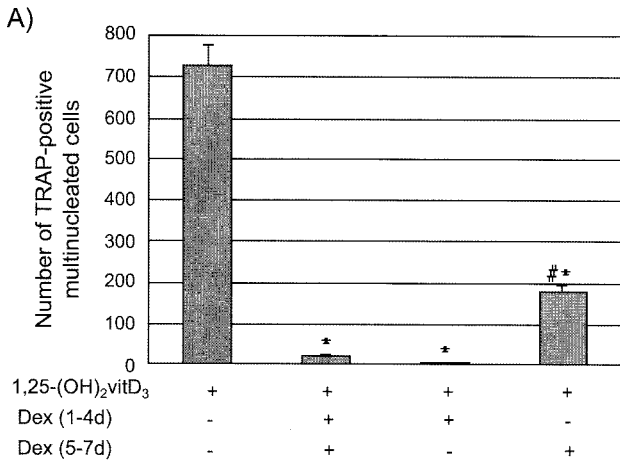
VNR was down-regulated by DEX during coculture

To identify whether DEX alters characteristics of osteoclast differentiation, we observed the expression pattern of various osteoclast differentiation markers. The expression of most osteoclast markers was not changed significantly by DEX except for two genes, the VNR β_3 subunit and CTR. CTR expression was up-regulated by DEX (Fig. 4A). A gradual increase in β_3 integrin expression in accordance with the progression of osteoclast differentiation was suppressed by DEX (Fig. 4A & B). As shown in Fig. 4C, DEX down-regulated $1,25\text{-}(OH)_2vitaminD_3$-induced β_3 subunit protein expression but not that of the α_v subunit. Although VNR is expressed in osteoblast lineage cells, VNR is normally enriched in osteoclasts (Hultenby *et al.*, 1993; Ross and Teitelbaum, 2005). Since we used a coculture system, it was necessary to examine the phenotype of cells that expressed VNR proteins. As shown in Fig. 5, the cells that stained brightly with anti-β_3 integrin antibody also stained positively with anti-mouse CD14 antibody, indicating that macrophage/monocyte

Fig. 2. Effect of dexamethasone on the formation of multinucleated osteoclasts according to the treatment period. Coculture were performd for 7 days in the presence of 1, $25\text{-}(OH)_2vitaminD_3$. Dexamethasone ($10^{-7}$ M) was added to the culture for the indicated period. TRAP staining was carried out and the number of TRAP (+) cells containing 3 or more nuclei were counted. A) Data represent means ± S.E. (N=6). * p<0.01, compared to control (1, $25\text{-}(OH)_2vitaminD_3$ alone), # p<0.01, compared to 1, $25\text{-}(OH)_2vitaminD_3$ + dexamethasone for 7 days. B) Original magnification ×40.
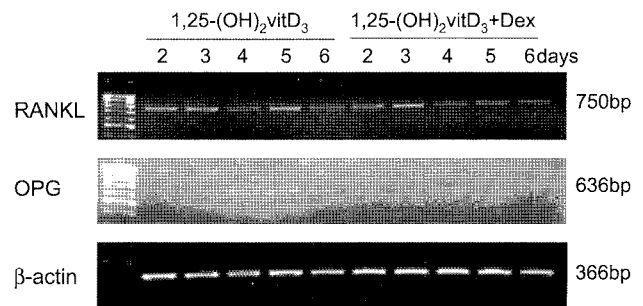

Fig. 3. Effect of dexamethasone on the expression of RANKL and OPG in coculture. Osteoblastic cells and bone marrow cells were cocultured with or without dexamethasone (10^{-7} M) in the presence of 1, $25\text{-}(OH)_2vitaminD_3$. After indicated culture period, total RNAs were isolated and RT-PCR was performed.
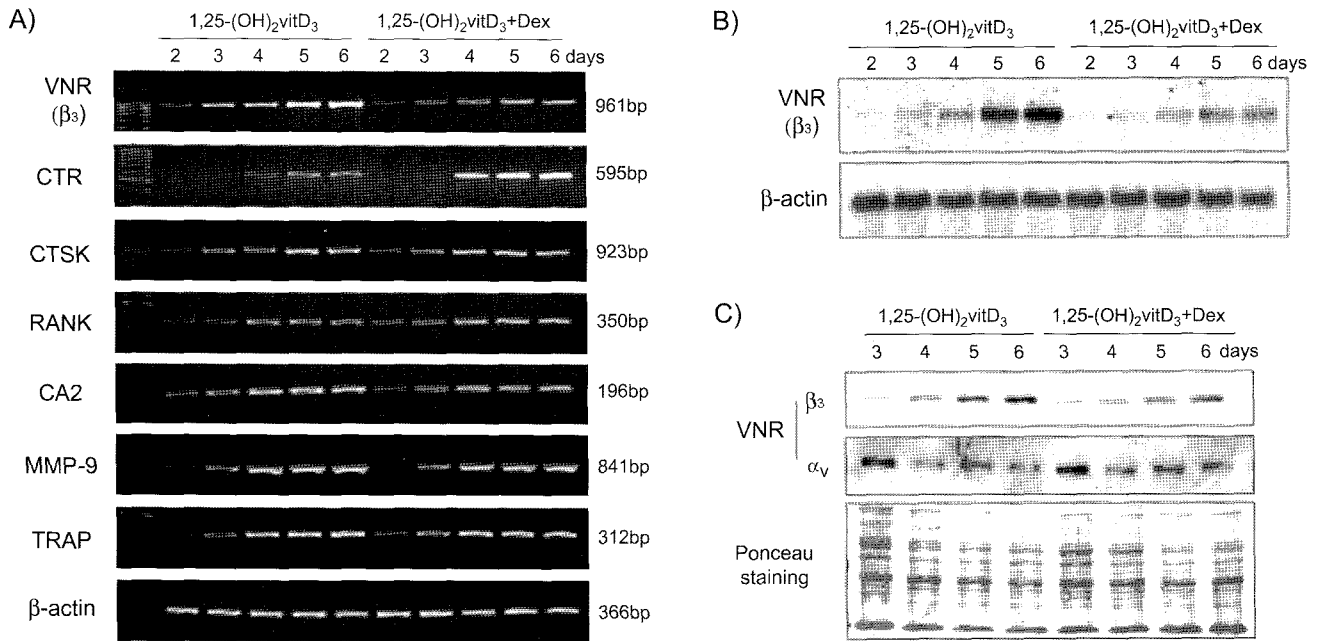
Fig. 4. Effect of dexamethasone on the expression of osteoclast differentiation markers. Osteoblastic cells and bone marrow cells were cocultured with or without dexamethasone $(10^{-7}$ M) in the presence of 1, 25-$(OH)_2$vitaminD_3. After indicated culture period, total RNAs or cell lysates were prepared and RT-PCR (A), northern blot analysis (B), or western blot analysis (C) was performed.
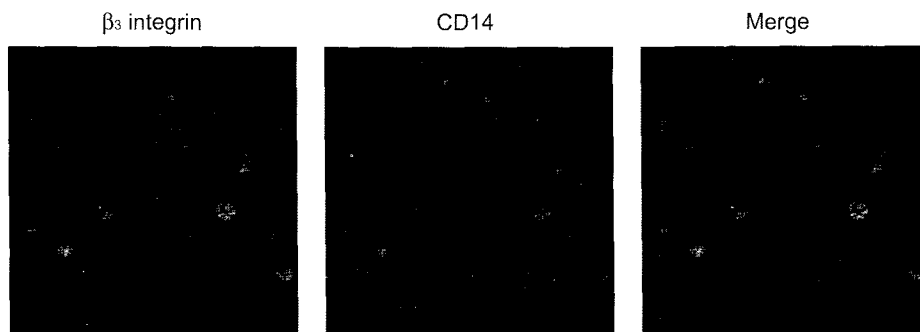
Fig. 5. Expression of β_3 integrin in CD14 (+) macrophage-monocyte lineage cells in coculture. Osteoblastic cells and bone marrow cells were cocultured for 4 days in the presence of 1, 25-$(OH)_2$vitaminD_3. Cells were then subjected to immunofluorescent double staining employing anti-β_3 antibody and anti-CD14 antibody. Original magnification ×200.

lineage cells mainly expressed β_3 integrin in this coculture system.

VNR blocking inhibited the formation of multinucleated osteoclasts

Next, coculture was performed with echistatin to elucidate the role of VNR in the formation of multinucleated osteoclasts. Echistatin is an RGD-containing snake venom that is known to specifically bind to and block the function of the β_3 integrin subunit (Nakamura et al., 1998). As shown in Fig. 6A, echistatin treatment during the entire culture period significantly decreased the number of multinucleated osteoclasts. Echistatin did not block the formation of TRAP(+) mononuclear prefusion osteoclasts (Fig. 6B). Multinucleated osteoclast formation was not

significantly effected when echistatin was added for only the first 4 days, but was severely inhibited by echistatin treatment during the last 3 days (Fig. 6A), implying that VNR plays a role in the multinucleation process during osteoclast differentiation.

DISCUSSION

In this study, we found that DEX inhibited TRAP(+) multinucleated osteoclast formation in a dose-dependent manner when added to a coculture of osteoblastic cells and bone marrow cells. It has been reported that DEX further enhances 1,25-$(OH)_2$vitaminD_3-induced RANKL expression in ST-2 cells (Yasuda et al., 1998). In addition, coculture of ST-2 cells and bone marrow cells in the

A)

1,25-(OH)2vitD3	+	+	+	+
Echlistatin (1-4d)	-	+	+	-
Echistatin (5-7d)	-	+	-	+

B)

1,25-(OH)2vitD$_3$ 1,25-(OH)$_2$vitD$_3$/Echistatin (1-7d)

1,25-(OH)$_2$vitD$_3$/Echistatin (1-4d)   1,25-(OH)$_2$vitD$_3$/Echistatin (5-7d)
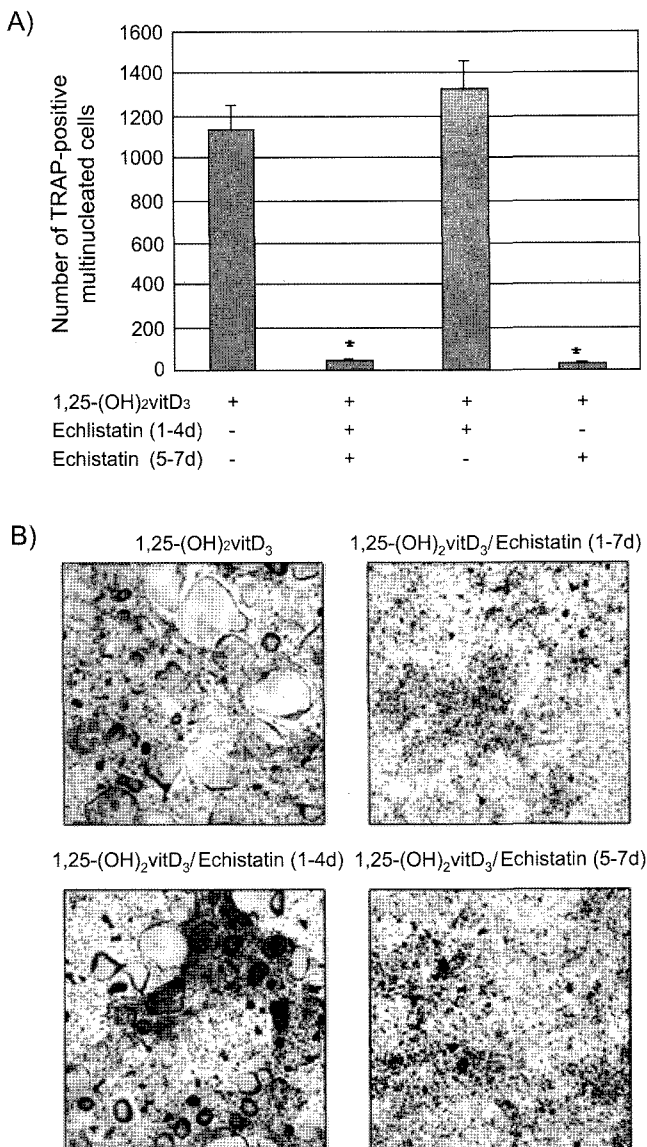
Fig. 6. Effect of echistatin on the formation of multinucleated osteoclasts. Osteoblastic cells and bone marrow cells were cocultured for 7 days in the presence of 1, 25-(OH)$_2$vitaminD$_3$. Echistatin (10^{-7} M) was added to the culture for indicated period. TRAP staining was carried out and the number of TRAP (+) cells containing 3 or more nuclei were counted. A) Data represent means ± S.E. (N=6). * p<0.01, compared to control (1, 25-(OH)$_2$vitaminD$_3$ alone). B) Original magnification ×40.

presence of 1,25-(OH)$_2$vitaminD$_3$ and DEX has produced multinucleated osteoclasts capable of resorbing bone (Nagai and Sato, 1999; Feng *et al.*, 2001). We used primary osteoblastic cells instead of ST-2 stromal cells, but we are unable to explain why the use of cells in a different stage of differentiation led to inhibition of osteoclastogenesis in the presence of DEX.

In this study, the expression of RANKL and OPG in coculture was not effected by the presence of DEX, implying that RANKL and OPG might not be the factors

responsible for the inhibition of TRAP(+) multinucleated osteoclast formation by DEX. This result is consistent with the observation that expression levels of osteoclast differentiation marker genes other than β_3 integrin were not suppressed by DEX.

Osteoclast formation consists of several steps, including recruitment of osteoclast progenitors, commitment into osteoclast precursors, fusion into multinucleated cells, and activation (Duong and Rodan, 2001). In this study, DEX blocked the formation of TRAP(+) multinucleated osteoclasts, but not that of TRAP(+) mononuclear cells. These results suggest that DEX might not influence the commitment of osteoclast progenitor cells into TRAP(+) osteoclast precursors or proliferation of osteoclast precursors. DEX might instead influence early osteoclast differentiation, which plays an essential role in the fusion of mononuclear prefusion osteoclasts during the later period of differentiation.

Inoue *et al.* (1998) found that uncommitted osteoclast precursors in the form of bone marrow macrophages contain an abundance of the integrin $\alpha_v\beta_5$, and that upon exposure to RANKL and the appearance of osteoclast-specific proteins, these cells cease to express $\alpha_v\beta_5$, which is replaced by VNR. We also saw that the expression of β_3 integrin mRNA and protein gradually increased as osteoclast differentiation proceeded but this gradual increase was down-regulated by DEX. The cells expressing β_3 integrin were largely macrophage/monocyte lineage cells in our coculture system. Expression of α_2, α_5, and α_v integrin chains shows consistent DEX-induced changes in primary human trabecular meshwork cells (Dickerson *et al.*, 1998), while the expression of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and β_5 did not significantly increase after DEX treatment in A549 alveolar epithelial cells (Sexton *et al.*, 2001). This study is the first showing DEX regulation of β_3 integrin during the differentiation of osteoclasts.

β_3 integrin plays a role in the function of osteoclasts but not in the formation of mature osteoclasts, as demonstrated by the observation that β_3 integrin mutation or knockout impairs bone resorptive activity of osteoclasts but not osteoclast formation itself (McHugh *et al.*, 2000; Feng *et al.*, 2001). Antibody to β_3 integrin inhibited osteoclast-mediated bone resorption in the thyroparathyroidectomized rat (Crippers *et al.*, 1996). However, we found that echistatin almost completely blocked multinucleated osteoclast formation. The inhibitory effect of echistatin was observed only in cells that expressed sufficient levels of VNR and were ready to fuse. This result is consistent with a previous report showing that echistatin specifically binds to β_3 integrin and inhibits the migration of prefusion osteoclasts and, eventually, the formation of multinucleated osteoclast-like cells (Nakamura *et al.*, 1998). Accordingly, down-regulation of β_3 integrin by DEX could be one of the

mechanisms mediating DEX inhibition of TRAP(+) mult-inucleated osteoclast formation. From these findings, we suggest that DEX treatment for the first 4 days of culture can suppress the induction of β_3 integrin expression, which might be sufficient to prevent TRAP(+) mononuclear osteoclast precursors from fusing to form multinucleated osteoclasts. If the continuing induction of β_3 integrin is necessary for ongoing multinucleation of the remaining mononuclear osteoclast precursors, DEX treatment during the last 3 days of culture would be expected to partially reduce multinucleated osteoclast formation.

The mechanism by which DEX down-regulates β_3 integrin was not explored in this study. The coculture system manifests a complex network of osteoblastic cells and bone marrow cells, making it difficult to understand which cells are primarily influenced by DEX to down-regulate β_3 integrin expression. Sustained ERK activity has recently been associated with β_3 integrin induction and subsequent cell surface expression of VNR during osteoclast differentiation (Kim et al., 2003). In addition, the same group has shown that inhibition of protein kinase C-β in osteoclast precursor cells abolished ERK and MEK activation and the subsequent differentiation and fusion processes of osteoclastogenesis by RANKL and macro-phage-colony stimulating factor (Lee et al., 2003). Although protein levels of PKC-βI and -βII were not significantly reduced by DEX in this study, the level of phosphorylated-protein kinase C-β did decrease significantly in the presence of DEX, suggesting the possibility that suppression of protein kinase C-β activity in osteoclast precursors might be involved in the inhibition of β_3 integrin expression by DEX (data not shown). At the same time, it cannot be ruled out that DEX inhibition of β_3 integrin expression could be mediated by its indirect action via osteoblastic cells, as DEX has had different effects on osteoclast formation according to the cell types used in coculture as a source of RANKL and other osteoclastogenic factors.

Taken together, our findings imply that DEX inhibits the formation of multinucleated osteoclasts, at least in part, through the down-regulation of β_3 integrin, which plays an essential role in the fusion of osteoclast precursors.

ACKNOWLEDGEMENTS

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