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Divergent Regulation of 1,25-Dihydroxyvitamin D_3 on Human Bone Marrow Osteoclastogenesis and Myelopoiesis

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Abstract The physiologically active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has influence over osteoclastogenesis and myelopoiesis, but the regulational mechanism is not well-defined. In this report, formation of osteoclast-like (OCL) cells from primitive myeloid colony-forming cells (PM-CFC) as mediated by $1,25(OH)_2D_3$ was examined. Our results present in this report clearly show that $1,25(OH)_2D_3$ dose-dependently stimulated OCL cell formation when added to suspension cultures of individually replated PM-CFC colonies. Marrow cells were plated with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or the human bladder carcinoma cell line 5637 conditioned medium (5637 CM) as the source of colony-stimulating activity. The $1,25(OH)_2D_3$ effect of osteoclast differentiation was associated with a concomitant decrease in clonogenic growth of myelopoietic progenitors in response to colony-stimulating activity. Secondly, the effect of adding the known stimulator of hematopoiesis, interleukin-1β (IL-1β) and/or $1,25(OH)_2D_3$ on human myeloid colony growth was assessed. IL-1β enhanced the formation of primitive myeloid colonies in response to GM-CSF by 160%. On the other hand, $1,25(OH)_2D_3$ dose-dependently inhibited both GM-CSF- and 5637 CM-driven myeloid colony formation by as much as 90% at 100 nM. Addition of IL-1β to GM-CSF-stimulated cultures dampened the inhibitory effect of $1,25(OH)_2D_3$. The inhibition of myeloid clonogenic growth by $1,25(OH)_2D_3$ was almost abolished (89%) by simultaneously adding anti-tumor necrosis factor-α monoclonal antibody (anti-TNF-α MoAb) to the culture medium. These results collectively suggest divergent roles for $1,25(OH)_2D_3$ in osteoclastogenesis and myelopoiesis, promoting the differentiation of OCL cells from primitive myeloid cells but inhibiting the proliferation of later myeloid progenitor cells. This inhibition of myeloid progenitors may be mediated by TNF-α. J. Cell. Biochem. 72:387–395, 1999.

Key words: hematopoietic progenitor; IL-1; TNF-α; osteoclast-like cell; differentiation; proliferation

Osteoclasts are derived from myelomonocytic bone marrow progenitor cells under the influence of hematopoietic cytokines and $1,25(OH)_2D_3$. However, neither the regulation of osteoclastogenesis nor the exact cellular target of $1,25(OH)_2D_3$ action is well-defined. The effects of $1,25(OH)_2D_3$ on this process has been studied in a variety of experimental systems including bone marrow-stromal cell co-cultures, stromal cell free cultures, and long-term bone marrow cultures in an attempt to better study this question [Suda et al., 1997]. Clearly, work by Roodman and his associates in human long-term bone marrow and stromal cell-free cultures has shown that cells at relatively later differentiational states (granulocyte-macrophage colony-forming cells, GM-CFC) are responsive to the differentiational effects of $1,25(OH)_2D_3$ and will form osteoclast-like cells [MacDonald et al., 1987; Kurihara et al., 1991].

However, a body of work exists suggesting that $1,25(OH)_2D_3$ may also have effects on earlier myeloid progenitors. Work in leukemic cell lines has shown that $1,25(OH)_2D_3$ promotes the differentiation of the human promyelocytic line HL-60 along a monocytic pathway [Miyaura et al., 1981]. When $1,25(OH)_2D_3$ is continuously present in the long-term bone marrow cultures,

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a complete cessation of hematopoiesis occurs by week 4 [Dorshkind et al., 1989]. Using murine primary bone marrow cells enriched for early precursors by 5-FU pretreatment, we have shown that $1,25(OH)_2D_3$ downregulates the M-CSF receptor while inhibiting proliferation, supporting the concept that the steroid acts even upon these early cells [Perkins and Teitelbaum, 1991].

In addition to possible direct effects on cell proliferation and differentiation, $1,25(OH)_2D_3$ has also been shown to stimulate cytokine production, in particular that of tumor necrosis factor-α (TNF-α) [Bhalla et al., 1991; Higashimoto et al., 1995]. The linkage between $1,25(OH)_2D_3$ and TNF-α is intriguing as TNF-α has been shown to inhibit growth of myeloid progenitor cells when continuously present in the culture medium of low density long-term bone marrow cultures [Abboud et al., 1987; Herrman et al., 1988, Murphy et al., 1988]. Further studies have shown that TNF-α will stimulate the formation osteoclast-like cells in culture [Pfeilschifter et al., 1987; Abu-Amer et al., 1997] as well as enhancing osteoclastic bone resorption in vitro [Bertolini et al., 1986] and in vivo [Konig et al., 1988]. Taken together, these studies suggested that TNF-a production under the influence of $1,25(OH)_2D_3$ may play a role in mediating the divergent effects of the steroid on myelopoiesis and osteoclastogenesis.

In order to better define the role of $1,25(OH)_2D_3$ in human myeloid progenitor cell proliferation and differentiation into osteoclast-like cells, we utilized colony-derived osteoclast-like cell formation system. Primitive myeloid colony-forming cells (PM-CFC) were isolated at day 14 from primary human bone marrow cultures as colonies that were proliferating in response to 5637 conditioned medium (containing GM-CSF, G-CSF, IL-3, and IL-1). These cells were replated in the presence of $1,25(OH)_2D_3$ to determine if they had the capacity to differentiate into osteoclast-like cells. Once it was determined that osteoclast-like cells could form from the PM-CFC, we evaluated the anti-proliferative effect of $1,25(OH)_2D_3$ on clonogenic growth of myeloid colonies in the presence of a variety of growth factors, including GM-CSF or 5637 CM (with or without IL-1). Finally, we determined by antibody blocking experiments that $1,25(OH)_2D_3$ may be acting via a paracrine or autocrine effect of TNF-α to inhibit myeloid progenitor proliferation.

MATERIALS AND METHODS

Preparation of Nonadherent Mononuclear Bone Marrow Cells

Human bone marrow was obtained from rib sections of hematologically normal patients removed during thoracotomy. Bone marrow cells were suspended in Iscove's Modified Dulbecco's Medium (IMDM; Gibco, Grand Island, NY) and fractionated by density gradient separation on HISTOPAQUE-1077 (Sigma, St. Louis, MO). The mononuclear bone marrow cells (MBMC) were isolated, washed, and resuspended in IMDM supplemented with 20% fetal bovine serum (FBS; Gibco). Adherent cells were depleted by incubation of the MBMC suspension in tissue culture dishes (100 × 20 mm; Falcon, Lincoln Park, NJ) for 1 h at 37°C in a humidified atmosphere of 5% CO_2. The nonadherent MBMC were collected.

Immature PM-CFC Colony-Derived OCL Cell Formation Cultures

Nonadherent MBMC were plated under conditions to enhance the formation of osteoclast-like cells using a modification of previously described osteoclast formation cultures [Horton et al., 1985; Kurihara et al., 1990; Kurihara et al., 1991]. Briefly, nonadherent MBMC were suspended at a concentration of 2×10^5 cells/ml in IMDM containing 1.5% methylcellulose (4,000 centipoises, Dow Chemical Co., Midland, MI), 20% FBS, and 10% 5637 CM (kindly pretested and provided by Professor T. M. Dexter, Paterson Institute for Cancer Research, Manchester, UK) as the optimal source of colony-stimulating activity for primitive myeloid cell expansion. This semi-solid preparation (5 ml) was cultured in tissue culture dishes (100 × 20 mm) for 14 days at 37°C, 5% CO_2 to allow for the formation of large PM-CFC colonies as described previously [Testa, 1985, Murphy et al., 1988]. Large individual day-14 PM-CFC colonies were located by an inverted microscope, removed using a pipette tip, and resuspended as a single colony/well or at 10^3–10^4 cells/well in 48-well tissue culture clusters (Costar, Cambridge, MA) containing IMDM supplemented with 10% FBS, 10% horse serum (Gibco). Cultures were also treated with the indicated concentrations of $1,25(OH)_2D_3$ (a kind gift from Dr. M. Uskokovic, Hoffman-La Roche, Nutley, NJ) to stimulate osteoclast-like cell formation. After 20 days incubation at 37°C in 5% CO_2 with

weekly feedings and replacement of all added cytokines and steroids with replacement of half of the culture medium, cells were examined for the presence of osteoclastic characteristics as described below.

Characterization of Osteoclast-Like Cells

Cultured cells in multiwell plates were fixed with 4% paraformaldehyde (Sigma) at 4°C, for 30 min. For the detection of tartrate-resistant acid phosphatase (TRAP), the adherent cell layers were incubated at 20°C, in the dark, for 30 min to 2 h, in 4% hexazotized pararosaniline (Sigma), as a coupler, and 0.2% Naphthol AS-B1 phosphate (Sigma), as an artificial substrate. The specimens were incubated with Mayer's hematoxylin solution (Sigma) at 20°C for 5 min. Cross-reactivity with the osteoclast-specific murine MoAb 13C2 [Horton et al., 1985; Davies et al., 1989] was simultaneously used to further identify OCL cells. Fixed cells were incubated overnight at 4°C with murine MoAb 13C2 (generous gifts from Dr. M. Horton, Imperial Cancer Research Funds, London, UK). Negative controls included omission of 13C2 MoAb and addition of an irrelevant murine IgG. Reactivity was visualized using VECTASTAIN avidin-biotin complex-alkaline phosphatase kit (ABC-AP kit; Vector Laboratories, Burlingame, CA). It has been previously shown that multinucleated cells formed from bone marrow using a similar long-term human bone marrow system that progenitors that are also TRAP and react with osteoclast-specific antibodies will have other features of osteoclast-like cells including the capacity to resorb bone [Kurihara et al., 1990].

Myeloid Colony Assays

The standard assay for bone marrow myeloid colony formation was carried out according to the method described elsewhere [Testa, 1985]. In brief, human nonadherent MBMC (2×10^5) were resuspended in IMDM (1 ml) with FBS (20%) in 0.33% agar (Difco Laboratories, Detroit, MI), using either 20 U/ml recombinant human (rhu) GM-CSF (a generous gift from Dr. H. P. Kocher, SANDOZ, Switzerland) or 10% 5637 CM as the source of colony-stimulating activity. Triplicate cultures plated in 35×10 mm tissue culture dishes were incubated at 37°C in 5% CO_2. The indicated concentrations of $1,25(OH)_2D_3$, rhuIL-1β (a kind gift from Glaxo Group Research, Ltd, UK), and anti-TNF-α MoAb (a kind gift of Dr. G. R. Adolf, Boehringer Ingelheim, Germany) were added to the myeloid colony cultures at the time of plating and maintained by weekly feedings for the duration of the experiment. Negative controls included omission of the cytokines/antibodies tested and addition of a same class, irrelevant immunoglobubin. In addition, some of the 5637 CM-stimulated cultures were supplemented at the beginning with 20 U/ml recombinant human erythropoietin (rhuEPO) (Cilag Ltd, UK) to allow development of mixed lineage hematopoietic cells, including erythrocytes. Colonies consisting of more than 50 cells were scored on day 9.

Statistics

Data were analyzed for statistical differences using one factor analysis of variance (ANOVA), followed by a Fisher PLSD multiple-range test. Values are expressed as the mean ± standard error of the mean (SEM) from at least two repeated experiments. Differences were considered significant if $P < 0.05$.

RESULTS

OCL Cell Formation Depends on Addition of $1,25(OH)_2D_3$

In this study, OCL cells were defined as bi- and multi-nucleated cells (BNC and MNC), of which 97.9% ± 1.5% exhibited TRAP positivity and 94.5% ± 2.7% cross-reacted with osteoclast-specific MoAb 13C2. None of the cells in the day-14 PM-CFC colonies demonstrated either TRAP activity or 13C2 reactivity. In the absence of exogenously added $1,25(OH)_2D_3$, low but variable numbers of BNC and MNC were formed only after an 18–20 day incubation. The results presented here clearly showed that $1,25(OH)_2D_3$ dose-dependently enhanced BNC and MNC formation (Fig. 1). The $1,25(OH)_2D_3$ stimulated BNC and MNC formation in the replated single-colony cultures after about 8 days, with BNC and MNC numbers increasing until about day 20. The minimum concentration of $1,25(OH)_2D_3$ required to stimulate OCL cell formation was approximately 0.1 nM, with this stimulatory effect peaking between 1–10 nM and a slight decrease in efficacy at 100 nM. The maximum MNC formation was increased more than two-fold compared to medium control, while a 1.7-fold increase in BNC formation was observed.

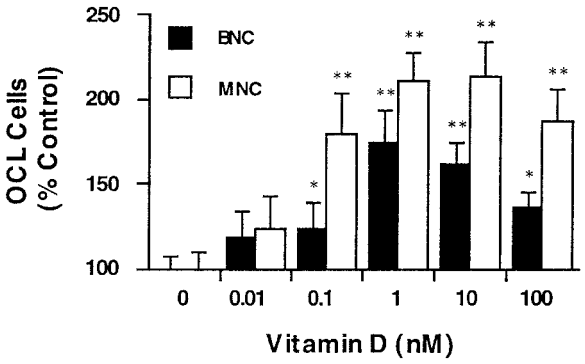

Fig. 1. Effect of increasing concentrations of $1,25(OH)_2D_3$ (1,25D) on the human OCL cell formation from day-14 PM-CFC colonies. Isolated day-14 PM-CFC colonies were cultured with increasing concentrations of $1,25(OH)_2D_3$. After 20 days, TRAP-positive bi- and multi-nucleated cells (BNC and MNC) were counted. Each bar represents the mean ± SEM of BNC or MNC appearing in each culture, expressed as a percentage of medium control. The experiment was repeated four separate times and bars are the average values of 10 replicate independent well cultures (*denotes $P < 0.05$, compared to medium control; **denotes $P < 0.01$, compared to medium control).

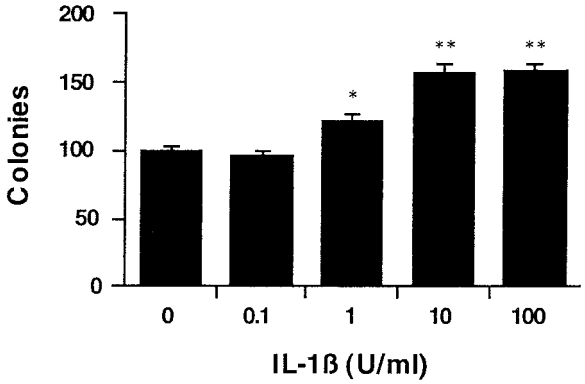

Fig. 2. Dose-dependent effect of rhuIL-1β on rhuGM-CSF supported human myeloid colony formation. The different indicated concentrations of rhuIL-1β were added to 2×10^5/ml mononuclear nonadherent marrow cells with a constant dose of 20 units/ml rhuGM-CSF at the time of plating. Control plates had no added rhuIL-1β. The cells were cultured for 9 days and the number of myeloid colonies ennumerated. The data is presented as the mean number of colonies ± SEM from four individual replicate independent cultures (*denotes $P < 0.05$, compared to control; **denotes $P < 0.01$, compared to control).

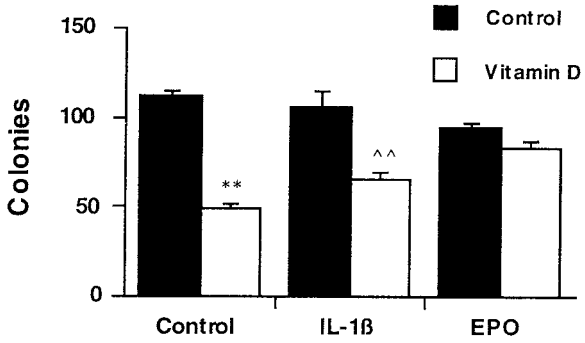

Fig. 3. Effect of different factors on human myeloid colony formation stimulated by 5637 CM. Ten units/ml rhuIL-1β, 20 units/ml erythropoietin or 10 nm $1,25(OH)D_3$ was added to the culture medium at the time of plating. Mononuclear nonadherent marrow cells (2×10^5/ml) were cultured for 9 days. A group of culture dishes supplemented with 5637 CM (10%) was used as a control. The data were expressed as mean number of colonies ± SEM from at least six replicate independent cultures (**denotes $P < 0.01$, compared to control;^^ denotes $P < 0.01$, compared to rhuIL-1β (10 U/ml); NS denotes not significant, compared to rhuEPO (20 U/ml).

Synergism between IL-1β and CSFs in Promotion of Myeloid Colony Formation

In order to drive the proliferation and differentiation of myeloid progenitor cells, rhuIL-1β, 5637 CM (a conditioned medium at least including IL-1β and GM-CSF that has been widely used in human long-term bone marrow cultures) or rhuGM-CSF were used to stimulate myeloid colony formation in a semi-solid culture system. IL-1β, a known promotor of primitive colony growth, by itself failed to promote any myeloid colony growth, while rhuGM-CSF and 5637 CM stimulated the formation of 99 ± 3 and 113 ± 2 myeloid colonies from 2×10^5 MBMCs, respectively. Addition of rhuIL-1β to rhuGM-CSF-stimulated cultures, however, dose-dependently increased both the size and number of myeloid colonies formed after 9 days of culture. This IL-1β-mediated enhancement was secondary to the formation of more primitive myeloid colonies, particularly between 1–100 U/ml rhuIL-1β (Fig. 2). On the other hand, addition of rhuIL-1β to 5637 CM-stimulated cultures resulted in no further increase in myeloid colony formation, probably reflecting the presence of IL-1β in the 5637 CM (Fig. 3).

Inhibition by $1,25(OH)_2D_3$ of Myeloid Colony Formation

In myeloid colony forming assays, the continuous presence of $1,25(OH)_2D_3$ dose-dependently inhibited the clonogenic growth of myeloid colonies. Addition of 10 nM $1,25(OH)_2D_3$ reduced myeloid clonogenic growth more than 55%, regardless of the colony-forming stimulus tested (Figs. 3,4). The dose-dependent inhibition of clonogenic proliferation by $1,25(OH)_2D_3$ in 5637 CM-stimulated cultures (Fig. 5) paralleled that found in the GM-CSF/IL-1β-induced myeloid cultures (Fig. 6). Significant inhibition was observed at doses as low as 0.01 nM $1,25(OH)_2D_3$

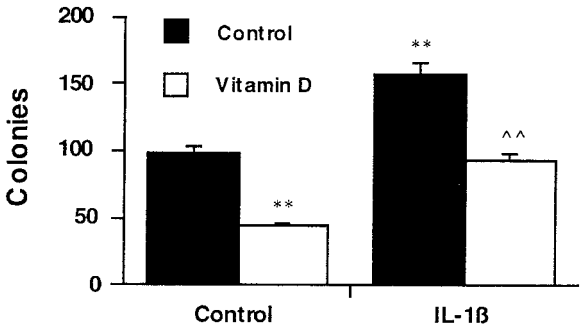

Fig. 4. Effect of rhuIL-1β and 1,25(OH)$_2$D$_3$ on human myeloid colony formation. Mononuclear nonadherent cells (2×10^5) per ml were cultured in the presence of rhuGM-CSF (20 U/ml) in the semi-solid system as control. 10 nM 1,25(OH)D$_3$ or 10 units/ml rhuIL-1β were added as indicated. The myeloid colonies were observed on day 9 of the culture. The data were expressed as mean number of colonies ± SEM from 10 independent replicate cultures (**denotes $P < 0.01$, compared to control; ^^ denotes $P < 0.01$, compared to rhuIL-β).

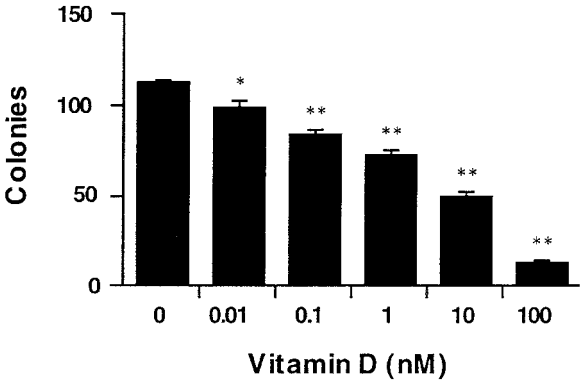

Fig. 5. Effect of increasing concentrations of 1,25(OH)$_2$D$_3$ on human myeloid colonies cultured in the presence of 5637 CM (10%). Mononuclear nonadherent marrow cells (2×10^5/ml) were cultured for 9 days in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$ and the number of myeloid colonies ennumerated. Cultures in which 1,25(OH)$_2$D$_3$ was absent served as a control. The data were expressed as mean number of myeloid colonies ± SEM from at four replicate independent cultures (*denotes $P < 0.05$, compared to control; **denotes $P < 0.01$, compared to control).

in 5637 CM-stimulated cultures and 0.1 nM in GM-CSF/IL-1β-stimulated cultures. Compared to control cultures, maximal inhibition was seen with 100 nM 1,25(OH)$_2$D$_3$, reducing 5637 CM- and GM-CSF/IL-1β-stimulated myeloid colony formation 89.1% and 62%, respectively (Figs. 5,6).

As observed above, rhuIL-1β promoted both the size and number of primitive myeloid colonies when co-cultured with rhuGM-CSF. Interestingly, rhuIL-1β reduced the inhibitory effect of 1,25(OH)$_2$D$_3$ on clonogenic proliferation from

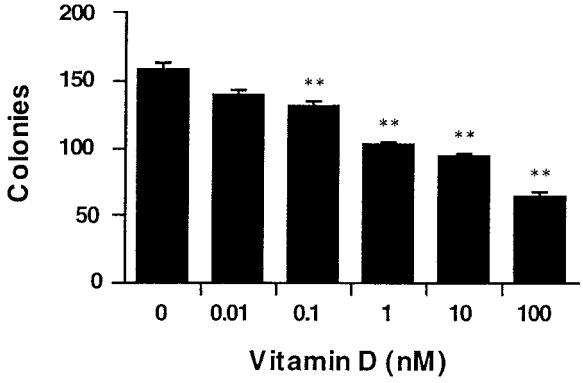

Fig. 6. Dose-response curve of human myeloid colony formation after addition of 1,25(OH)$_2$D$_3$ in the presence of rhuGM-CSF (20 U/ml) and rhuIL-1β (10 U/ml). All factors were added to the culture medium at the time of plating. Mononuclear nonadherent marrow cells (2×10^5/ml) were cultured for 9 days. Culture without the addition of 1,25(OH)$_2$D$_3$ served as a control. The data were expressed as mean number of myeloid colonies ± SEM from six replicate independent cultures (**denotes $P < 0.01$, compared to control).

55.6% for GM-CSF-stimulated cultures to 40.7% for GM-CSF-stimulated cultures with rhuIL-1β (Fig. 4). Similarly, 1,25(OH)$_2$D$_3$ produced a 38% reduction of the myeloid colony formation in response to the combination of 5637 CM and rhuIL-1β, as compared to a 56.1% inhibition in the cultures with 5637 CM alone (Fig. 3). Less inhibition of growth by 1,25(OH)$_2$D$_3$ was seen with higher doses of IL-1β, suggesting a dose-dependent mechanism. This suggests that primitive colonies induced by GM-CSF plus rhuIL-1β were less sensitive to the effect of 1,25(OH)$_2$D$_3$ on anti-clonogenic proliferation, implying that IL-1 may be able to modulate the effects of 1,25(OH)$_2$D$_3$ on the proliferation of primitive cells. In addition, 1,25(OH)$_2$D$_3$ had no significant effect on mixed lineage-CFC colony formation induced by 5637 CM in combination with rhuEPO (Fig. 3).

Inhibition by 1,25(OH)$_2$D$_3$ of Myeloid Colony Formation Is Mediated by TNF-α Secretion

Addition of anti-TNF-α MoAb or an irrelevant IgG alone had no significant effect on myeloid colony formation in response to 5637 CM in this culture system (Fig. 7). As stated earlier, 1,25(OH)$_2$D$_3$ produced a significant inhibition of myeloid colony formation, however, these anti-proliferative effects of 1,25(OH)$_2$D$_3$ were reversed in the presence of a neutralizing MoAb to TNF-α, following a sigmoidal dose response curve. To release the inhibition of my-

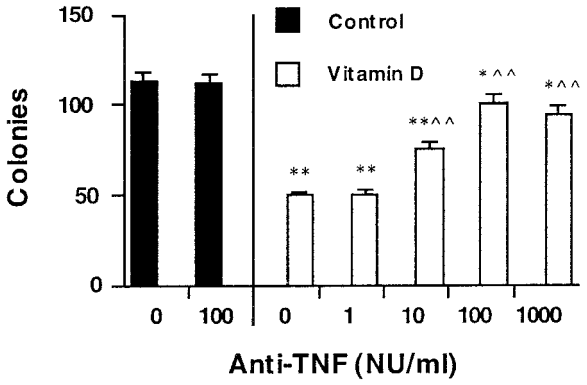

Fig. 7. Effect of anti-TNF-α MoAb on inhibition of myeloid colony formation by $1,25(OH)_2D_3$. All factors were added to the culture dishes at the time of seeding. Mononuclear nonadherent marrow cells (2×10^5/ml) were cultured in the presence of 10 nm $1,25(OH)_2D_3$, 10% 5637 CM and the indicated levels of anti-TNF for 9 days. Controls included 10% 5637 CM only (control) and 100 units of anti-TNF without addition of 10 nm $1,25(OH)_2D_3$. The data were expressed as mean myeloid colony numbers ± SEM from at 10 replicate independent cultures (*denotes $P < 0.05$, compared to control; **denotes $P < 0.01$, compared to control; ^^ denotes $P < 0.01$, compared to $1,25(OH)_2D_3$ alone).

eloid colony formation induced by $1,25(OH)_2D_3$, the minimal concentration of anti-TNF-α MoAb was approximately 10 NU/ml. The maximal abolition (89%) of $1,25(OH)_2D_3$ inhibition occurred at about 100 NU/ml of anti-TNF-α MoAb (Fig. 7). This suggests that $1,25(OH)_2D_3$'s effects on proliferation may be, in part, mediated through TNF-α.

DISCUSSION

Myeloid and osteoclast precursor cells require a source of colony stimulating activity, including G-CSF, IL-3, GM-CSF, and M-CSF, in order to proliferate, depending upon maturational stage of differentiation. Other factors such as IL-1, IL-6, and SCF can synergistically enhance proliferation of myeloid cells under the influence of colony stimulating factors (CSFs). As mentioned above, work in the bone marrow-stromal cell co-cultures and stromal cell free cultures has examined OCL cell formation from GM-CFC, cells at a later myeloid stage of differentiation as stimulated by GM-CSF. Our treatment of bone marrow cells with GM-CSF yielded similar colonies of mature granulocytes and macrophages. However, co-incubation of IL-1β with GM-CSF led to the formation of larger colonies of less differentiationally mature cells than GM-CFC. Likewise, colonies formed by stimulation with 5637 CM only, a conditioned medium containing both GM-CSF and IL-1β [Gabrilove et al., 1986; McNiece et al., 1989], resembled GM-CSF/IL-1β-stimulated colonies in both their larger size and composition of less mature cells than observed with GM-CFC, confirming that this experimental treatment favors formation of more immature myeloid precursor cells. Interestingly, addition of IL-1β to 5637 CM-stimulated cultures resulted in no further enhancement in colony growth, implying saturation of the response to IL-1. In effect, IL-1 appears to stimulate myelopoiesis by increasing the proliferation of early myeloid precursors, thus increasing the size and decreasing the maturational level of colonies formed. It has also been suggested that IL-1 may act to stimulate myelopoiesis by up-regulating the myeloid cell receptors for colony stimulating factors [Zhou et al., 1988; Zsebo et al., 1988]. Thus, IL-1 is not a direct growth stimulus, but a synergistic growth factor that recruits primitive progenitor cells to a growth-factor-responsive state to form primitive myeloid colonies (larger colonies) in a semi-solid culture system [Bartelmez et al., 1989; Smith et al., 1991; Dinarello, 1996]. In addition, IL-1 may also alter the bone marrow microenvironment by increasing the production of cytokines such as IL-6 and IL-11, thereby altering primitive myeloid precursor proliferation and differentiation [Haynesworth et al., 1996]. Further work to more clearly define the role of IL-6 and IL-11 production as a mechanism for IL-1 effects on myeloid precursor proliferation and differentiation are currently in progress.

The anti-proliferative effect of $1,25(OH)_2D_3$ on myelopoietic-progenitor clonogenic growth was investigated using a semi-solid culture system. The addition of $1,25(OH)_2D_3$ produced a significant reduction of myeloid clonogenic growth in the cultures presented with GM-CSF or 5637 CM for 9 days. Previous reports have confirmed that the colonies formed on day 7–9 in cultures enriched with colony-stimulating activity such as GM-CSF or 5637 CM represent later stages of myeloid progenitor cells, mainly the granulocyte-macrophage lineage [Jacobsen et al., 1979; Testa, 1985]. This result indicates that $1,25(OH)_2D_3$ inhibits the proliferation of later stage myeloid progenitor cells. However, $1,25(OH)_2D_3$ may have less anti-proliferative effect on earlier stage myelopoietic progenitors. IL-1 added to myelopoietic progenitor cell cultures promoted primitive colony growth and

EPO stimulated early multilineage colony formation. When $1,25(OH)_2D_3$ was added to these cultures, there was much less or no inhibition of primitive or multilineage clonogenic proliferation compared with the responses observed in the later stage myeloid cultures. These data collectively suggest that $1,25(OH)_2D_3$ promotes the differentiation of OCL cells from early stage myelopoietic progenitor cells, including PM-CFC. The anti-proliferative effect of $1,25(OH)_2D_3$ is more pronounced in later stage myelopoietic progenitor cells than in earlier stages.

Osteoclasts are believed to be differentiated from precursor cells common to the myeloid line, whose end result includes monocytes and macrophages. While the pre-osteoclast must branch off before terminal differentiation into the macrophage, it remains unclear what minimum stage of myeloid development is necessary before shunting into the osteoclast developmental pathway. OCL cells have been successfully formed from cultures stimulated with either GM-CSF or M-CSF; however, studies utilizing earlier myeloid precursors is much less common. In the present study, the stromal-osteoblastic cell free colony-derived OCL cell formation system has been used to examine the regulation of OCL cell formation in the presence of $1,25(OH)_2D_3$ to exclude effects of $1,25(OH)_2D_3$ and the cytokines examined herein upon the stromal/osteoblastic cells persistent within long-term bone marrow cultures. The results demonstrated that $1,25(OH)_2D_3$ greatly enhanced the formation of OCL cells containing two or more nuclei from primitive myeloid precursors. At concentrations between 1 nM and 10 nM of $1,25(OH)_2D_3$, maximal differentiation of primitive myeloid precursors into osteoclast-like cells occurred, similar to observations by MacDonald et al. [1987] utilizing modified long-term bone marrow cultures. Analysis of OCL cells showed $1,25(OH)_2D_3$ produced a significantly higher rate of MNC formation over BNC formation. These results collectively indicate that $1,25(OH)_2D_3$, while promoting differentiation of primitive myeloid progenitor cells toward the osteoclastic lineage, may also promote cell fusion in the later stages of osteoclast development, as suggested previously by Fan et al. [1996]. It has been recently reported that $1,25(OH)_2D_3$ can induce osteoclastogenesis partially by modulation of M-CSF and IL-6 production [Sarma and Flanagan, 1996; Schiller et al., 1997], and osteoclasts can develop from pluripotent embryonic stem cells through a c-fms mediated pathway [Yamane et al., 1997]. Other experiments have demonstrated that $1,25(OH)_2D_3$ can also promote more mature circulating and cord blood monocytes into OCL cells under certain conditions [Fujikawa et al., 1996; Roux et al., 1996].

The major hormonally active metabolite of vitamin D_3, $1,25(OH)_2D_3$ has hemato-immunoregulatory potential, regulating both hematopoietic and immunological cells. The receptors of $1,25(OH)_2D_3$ have been demonstrated in human monocytic cells, activated T cells, myeloid cell lines, and bone marrow stromal cells [Provvedini et al., 1983; Kizaki et al., 1991; Bellido et al., 1993], but there is still no direct evidence showing the receptors of $1,25(OH)_2D_3$ expressed in the primitive as well as late myeloid progenitors. In addition, $1,25(OH)_2D_3$ affects cytokine production, including TNF-α [Bhalla et al., 1991; Higashimoto et al., 1995]. Inhibition of hematopoiesis by TNF-α has been shown by experiments demonstrating inhibition of clonogenic growth of normal human myeloid progenitor cells, when it is continuously present in the culture medium [Abboud et al., 1987; Murphy et al., 1988]. In this study, possible mechanisms of the anti-proliferative effect of $1,25(OH)_2D_3$ on late myelopoietic-progenitor clonogenic growth were explored by the simultaneous addition of neutralizing anti-TNF-α MoAb in the culture medium enriched with 5637 CM for 9 days. The inhibition by $1,25(OH)_2D_3$ of human myelopoietic colony formation was almost abrogated by the simultaneous presence of neutralizing anti-TNF-α MoAb in the culture medium. This reduction of the inhibitory effects on myelopoietic-progenitor clonogenic growth occurred maximally (89%) at a concentration of 100 NU/ml anti-TNF-α MoAb. Therefore, we infer that the inhibition of human myelopoietic-progenitor clonogenic proliferation by the addition of $1,25(OH)_2D_3$ is mediated by continuous autocrine/paracrine production of TNF-α from $1,25(OH)_2D_3$-treated progenitor and other accessory cells. It has been reported that $1,25(OH)_2D_3$ directly enhanced TNF-α secretion in the presence of LPS [Prehn et al., 1992; Abu-Amer and Bar-Shavit, 1994]. In addition, human studies also showed that after oral administration of $1\alpha(OH)D_3$ (a precursor of $1,25(OH)_2D_3$), lipopolysaccharide (LPS)-induced TNF-α production by blood mononuclear cells from the patient is significantly

higher than controls [Haran et al., 1994]. Interestingly, in contrast to its inhibitory effects on hematopoiesis, TNF-α has been reported to increase formation of OCL cells in the modified long-term bone marrow cultures [Pfeilschifter et al., 1989], and to stimulate osteoclastic bone resorption [Bertolini et al., 1986; König et al., 1988], suggesting that it may be a modulator of osteoclast formation, a concept that is supported by both in vitro and in vivo observations. TNF-α, via its p55 receptor, also mediates LPS-stimulated osteoclastogenesis [Abu-Amer et al., 1997], and TNF-α has been implicated as a pro-osteoclastogenic cytokine responsible for bone loss in estrogen deficiency states. The TNF receptor superfamily contains a novel factor, osteoprotegerin, that appears to regulate bone resorption [Kimble et al., 1996, 1997; Simonet et al., 1997].

In summary, we found that IL-1 synergized with lineage-specific CSFs to promote primitive myelopoietic progenitor colony formation in stromal cell free human bone marrow cultures. Addition of $1,25(OH)_2D_3$ promoted the differentiation of primitive myeloid lineage cells to the osteoclast pathway, stimulating fusion of mononucleated pre-osteoclast-like cells to form osteoclast-like BNC and MNC. Simultaneously, $1,25(OH)_2D_3$ inhibited the later stages of myelopoietic progenitor proliferation. Addition of antibodies against TNF-α partially reversed the effects of $1,25(OH)_2D_3$ on proliferation, suggesting that TNF-α production may be an important component of $1,25(OH)_2D_3$ activity. This data support the concept that $1,25(OH)_2D_3$ plays divergent roles in the regulation of osteoclastogenesis and myelopoiesis, inhibiting proliferation of myeloid progenitor cells, as well as promoting osteoclast differentiation. These divergent actions of $1,25(OH)_2D_3$ on the regulation of osteoclastogenesis and myelopoiesis appear to correlate with the divergent roles of TNF-α, which stimulate osteoclastogenesis but inhibit myelopoiesis, suggesting stimulating of TNF-α production by $1,25(OH)_2D_3$ provides a mechanism of $1,25(OH)_2D_3$ action on hematopoietic, osteoclastic precusors.

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