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Regulation of Osteocalcin Production and Bone Resorption by 1,25-Dihydroxyvitamin D_3 in Mouse Long Bones: Interaction with the Bone-Derived Growth Factors TGF-β and IGF-I*

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ABSTRACT

Bone cells produce multiple growth factors that have effects on bone metabolism and can be incorporated into the bone matrix. Interplay between these bone-derived growth factors and calciotropic hormones has been demonstrated in cultured bone cells. The present study was designed to extend these observations by examining the interactions between either transforming growth factor-β (TGF-β) or insulin-like growth factor-I (IGF-I) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in a mouse long bone culture model with respect to osteocalcin production and bone resorption. In contrast to the stimulation in rat and human, in the fetal mouse long bone cultures, $1,25(OH)_2D_3$ caused a dose-dependent inhibition of osteocalcin production. Both the osteocalcin content in the culture medium and in the extracts of the long bones was reduced by $1,25(OH)_2D_3$. This effect was not specific for fetal bone because $1,25(OH)_2D_3$ also reduced osteocalcin production by the neonatal mouse osteoblast cell line MC3T3. TGF-β inhibited whereas IGF-I dose-dependently increased osteocalcin production in mouse long bones. The combination of TGF-β and $1,25(OH)_2D_3$ did not result in a significantly different effect compared with each of these compounds alone. The IGF-I effect was completely blocked by $1,25(OH)_2D_3$. In the same long bones as used for the osteocalcin measurements, we performed bone resorption analyses. Opposite to its effect on osteocalcin, $1,25(OH)_2D_3$ dose-dependently stimulated bone resorption. TGF-β reduced and IGF-I did not change basal (i.e., in the absence of hormones) bone resorption. Our results show that $1,25(OH)_2D_3$-enhanced bone resorption is dose-dependently inhibited by TGF-β and IGF-I. Regression analysis demonstrated a significant negative correlation between $1,25(OH)_2D_3$-induced bone resorption and osteocalcin production. The specificity for their effect on $1,25(OH)_2D_3$-stimulated bone resorption was assessed by testing the effects of TGF-β and IGF-I in combination with parathyroid hormone (PTH). Like $1,25(OH)_2D_3$, PTH dose-dependently stimulates bone resorption. However, PTH-stimulated bone resorption was not affected by TGF-β. Like $1,25(OH)_2D_3$-stimulated bone resorption, IGF-I inhibited the PTH effect but at a 10-fold higher concentration compared with $1,25(OH)_2D_3$. In conclusion, the present study demonstrates growth factor–specific interactions with $1,25(OH)_2D_3$ in the control of osteocalcin production and bone. With respect to bone resorption, these interactions are also hormone specific. The present data thereby support and extend the previous observations that interactions between $1,25(OH)_2D_3$ and bone-derived growth factors play an important role in the control of bone metabolism. These data together with the fact that TGF-β and IGF-I are present in the bone matrix and potentially can be released during bone resorption support the concept that growth factors may control the effects of calciotropic hormones in bone in a localized and possibly temporal manner. Finally, in contrast to human and rat, in mice $1,25(OH)_2D_3$ reduces osteocalcin production and this reduction is paralleled by stimulation of bone resorption by $1,25(OH)_2D_3$. These data thereby show a dissociation between osteocalcin production and bone resorption. (J Bone Miner Res 1998;13:36–43)

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INTRODUCTION

The steroid hormone 1,25-dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) and the polypeptide hormone parathyroid hormone (PTH) are important systemic regulators of calcium homeostasis and skeletal metabolism.[1,2] In response to a decrease in serum calcium concentration, $1,25(OH)_2D_3$ stimulates calcium absorption in the intestine and stimulates bone resorption. PTH increases serum calcium levels by increasing renal 1α-hydroxylase activity, renal calcium reabsorption, and bone resorption. Bone cells have been shown to produce a wide array of proteins, including growth factors, which have effects on bone metabolism and can be deposited in the bone matrix. Among these are members of the transforming growth factor-β (TGF-β) superfamily and insulin-like growth factors (IGFs), which affect osteoblast cell replication and differentiation.[3] TGF-β is a stimulator of bone formation, which is locally produced by osteoblasts[4] and osteoclasts[5] and is secreted and stored in an inactive form in bone.[6,7] TGF-β can be released from the extracellular matrix during bone resorption by osteoclasts[5] and it modulates the expression of several markers of the osteoblast phenotype.[8-15] Insulin-like growth factor-I (IGF-I) is produced by osteoblasts and stored in the bone matrix. IGF-I stimulates the proliferation and/or differentiation of osteoblasts and osteoclast progenitors.[16-18] IGFs can bind to IGF-binding proteins, the IGFBPs, which may either enhance or limit the effects of IGFs.[19] It is anticipated that these bone-derived growth factors do not act independently but interact with the calciotropic hormones. Data have become available indicating that systemic hormones affect the synthesis of local factors, which may be the ultimate mediators of their effects on bone metabolism.[20-22] In addition, the presence of local growth factors may regulate the eventual response to the calciotropic hormones at the target tissue/cell level.[23-25] We have recently shown that TGF-β strongly inhibits $1,25(OH)_2D_3$ induction of osteocalcin and osteopontin expression in osteoblasts via a block of the vitamin D receptor binding to the vitamin D responsive elements in the osteocalcin and osteopontin promoters.[26] The aim of the present study was to extend the observations on interaction between TGF-β and $1,25(OH)_2D_3$ in osteoblasts to osteocalcin production and bone resorption in a long bone culture model. In addition, growth factor and hormone specificity were examined by testing also IGF-I and PTH. These data may, thereby, provide further insight into the interaction between bone-derived growth factors and systemic regulators of bone metabolism, in particular $1,25(OH)_2D_3$. The present study provides support for the concept of local control by bone-derived growth factors of calciotropic hormones–regulated bone metabolism.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$ was kindly provided by LEO Pharmaceuticals BV (Weesp, The Netherlands). Rat PTH was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). TGF-β type 2 was generously provided by Dr. J. Feyen, Sandoz Pharma Ltd. (Basel, Switzerland). IGF-I and des-IGF-I were purchased from GroPep Ltd. (Adelaide, Australia). ^{45}Ca was purchased from Amersham International (Aylesbury, Buckinghamshire, U.K.). Fetal calf serum (FCS), Biggers-Gwatkin-Judah (BGJ) medium, penicillin, streptomycin, and glutamine were from Flow Laboratories (Irvine, Scotland, U.K.). All other reagents were of the best grade commercially available.

Mouse osteocalcin assay

Osteocalcin levels were measured in medium and extracts of fetal mouse long bone cultures and the osteoblast-like cell line MC3T3 according to the method of Prof R. Bouillon (personal communication).[27] Extracts of fetal mouse long bones were prepared either by the addition of 5% formic acid to radii/ulnae for 24 h, sonification and neutralization (pH 7.4) by the addition of NaOH, or by the addition of a 6 M guanidine-HCl/0.1 M Tris-buffer (pH 8.0) followed by sonification. MC3T3 cells were cultured in alpha modified essential medium (α-MEM) containing 10% FCS for 2 weeks until confluency and were subsequently incubated with $1,25(OH)_2D_3$ for 48 h. MC3T3 cells were extracted by the addition of a 6 M guanidine-HCl/0.1 M Tris-buffer (pH 8.0) followed by sonification.

Bone resorption assay

The effects of systemic and local factors on bone resorption were assessed by measuring the percentage of ^{45}Ca release from prelabeled 17-day-old fetal mouse radii and ulnae after 3 and 6 days of culture, a method that is based on the one described by Raisz.[28] Mice were injected subcutaneously with 30 μCi of ^{45}Ca on day 16 of gestation. Fetal mice were removed on day 17, and labeled radii/ulnae were dissected. Subsequently, radii/ulnae were cultured in 400 μl of BGJ medium with 5% charcoal-treated FCS at 37° C for 24 h to reduce free exchangeable calcium. Then the medium was refreshed and long bones were incubated with or without systemic factors ($1,25(OH)_2D_3$ or PTH) in the absence or presence of growth factors (TGF-β or IGF-I). After 3 days, medium was removed and replaced by medium containing the same test compound(s). After another 3 days, medium was removed and radii/ulnae were decalcified with 5% formic acid. The amount of ^{45}Ca was determined in medium collected after 3 and 6 days, as well as in the 5% formic acid extracts using a liquid scintillation counter and used to calculate the total ^{45}Ca content and the cumulative amount of ^{45}Ca released from radii/ulnae.

Statistical analysis

Interactions between various hormones and growth factors tested were statistically evaluated using an analysis of variance for two-way factorial design.[29] This method enables the significance of the interaction between different factors to be evaluated and was applied in all cases when two compounds were added together. The correlation between bone resorption and osteocalcin production was evaluated using linear regression analysis. All other statistical analyses were done by Student's *t*-test.

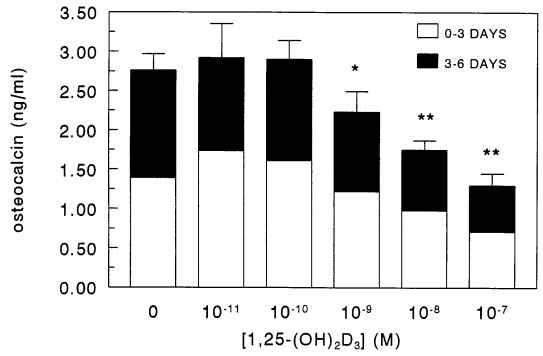

FIG. 1. The effect of $1,25(OH)_2D_3$ on the osteocalcin level in fetal mouse long bones. Fetal mouse radii/ulnae were incubated with several concentrations of $1,25(OH)_2D_3$, and osteocalcin levels were measured in the medium after 6 days. Data are presented as mean ± SEM of 8-50 cultures. $^*p < 0.002$, $^{**}p < 0.001$ vs. control.

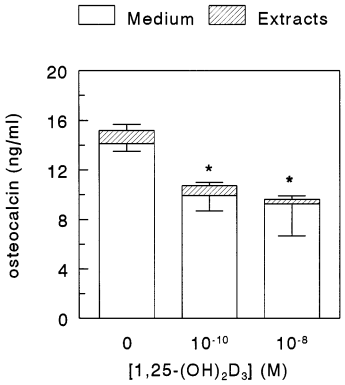

FIG. 2. The effect of $1,25(OH)_2D_3$ on the osteocalcin level in the mouse osteoblast-like cell line MC3T3. MC3T3 cells were cultured for 2 weeks until confluence, followed by the incubation with 10^{-10} M or 10^{-8} M $1,25(OH)_2D_3$ for 48 h. Osteocalcin levels were measured in medium as well as cell extracts of MC3T3 cells. Data are presented as mean ± SD of two cultures. $^*p < 0.05$ vs. control.

RESULTS

The effects of bone-derived growth factors and $1,25(OH)_2D_3$ on osteocalcin content and release into medium of cultured fetal mouse long bones

$1,25(OH)_2D_3$ is a potent stimulator of osteocalcin production by human and rat osteoblasts. In the present study, osteocalcin levels were measured after 3 and 6 days of incubation in the medium and extracts of fetal mouse radii and ulnae. $1,25(OH)_2D_3$ caused a dose-dependent reduction of the osteocalcin level. As shown in Fig. 1, after both 3 and 6 days, a reduction of osteocalcin concentration in the medium was observed. In extracts of the long bones, using two different extraction protocols, the amount of osteocalcin was detectable, but low, and no significant change was observed after incubation with $1,25(OH)_2D_3$ (data not shown). Thus, the reduction of osteocalcin in the medium was not the result of an increase in the radii and ulnae. This inhibitory effect of $1,25(OH)_2D_3$ on osteocalcin is contradictory to the stimulatory effect of $1,25(OH)_2D_3$ on osteocalcin production in rat and human osteoblast-like cells. To address specificity for the fetal mouse long bone system, we further investigated the effect of $1,25(OH)_2D_3$ on osteocalcin production in the neonatal mouse osteoblast-like cell line MC3T3. Similar to the long bone cultures, $1,25(OH)_2D_3$ reduced osteocalcin levels in medium as well as in cellular extracts of MC3T3 cells (Fig. 2).

In rat and human osteoblasts, TGF-β has an inhibitory effect on basal osteocalcin synthesis,[26,30] and recently we have demonstrated that in these cells TGF-β potently inhibits $1,25(OH)_2D_3$-stimulated osteocalcin production.[24,26] In mouse long bones, TGF-β dose-dependently strongly reduced the basal osteocalcin levels in long bone cultures. The inhibition by 10 ng/ml TGF-β was more potent than the inhibition by $1,25(OH)_2D_3$, about 70% and 40%, respectively (Fig. 3A). No significant interaction between $1,25(OH)_2D_3$ and TGF-β was detected with respect to the inhibition of osteocalcin. However, from 10 to 1000 ng/ml TGF-β, the inhibition was less pronounced in the presence than in the absence of $1,25(OH)_2D_3$ (Fig. 3A).

Specificity of these TGF-β effects was demonstrated by examining IGF-I. Unlike TGF-β, IGF-I caused an increase in osteocalcin (Fig. 3B), and at 100 and 1000 ng/ml IGF-I, a significant interaction with $1,25(OH)_2D_3$ was observed. The effect of the combination was similar to the effect of $1,25(OH)_2D_3$ alone, i.e., $1,25(OH)_2D_3$ blocks the induction by IGF-I.

Effects of local factors on the $1,25(OH)_2D_3$ and PTH enhancement of bone resorption

A prominent effect of $1,25(OH)_2D_3$ is stimulation of bone resorption. As shown in Fig. 4, $1,25(OH)_2D_3$ dose-dependently stimulates bone resorption in the fetal mouse radii/ulnae as measured by release of prelabeled ^{45}Ca. This effect could already be observed after 3 days (data not shown) and was most pronounced after 6 days of incubation. To examine hormone specificity of the bone- resorbing effects of the growth factors, besides $1,25(OH)_2D_3$, PTH was also studied. Like $1,25(OH)_2D_3$, PTH also dose-dependently stimulated bone resorption. At lower concentrations ($< 10^{-9}$ M), $1,25(OH)_2D_3$ seems to be more potent than PTH.

Incubation of fetal mouse long bones with TGF-β caused a small but significant inhibition of basal bone resorption after 6 days (Fig. 5). As shown in Fig. 5, after 6 days of culture, the induction of bone resorption by $1,25(OH)_2D_3$ (10^{-8} M) was inhibited in a dose-responsive manner by 10 and 100 ng/ml TGF-β. The effect of TGF-β after 3 days of incubation (data not shown) was similar but less pronounced compared with the effects after 6 days of incubation. Unlike $1,25(OH)_2D_3$, PTH-stimulated bone resorption was not changed by the presence of TGF-β.

IGF-I did not affect basal bone resorption of fetal mouse radii/ulnae after 3 (data not shown) and 6 days (Fig. 6). However, in combination with $1,25(OH)_2D_3$, IGF-I caused a dose-dependent inhibition of $1,25(OH)_2D_3$ stimulation of

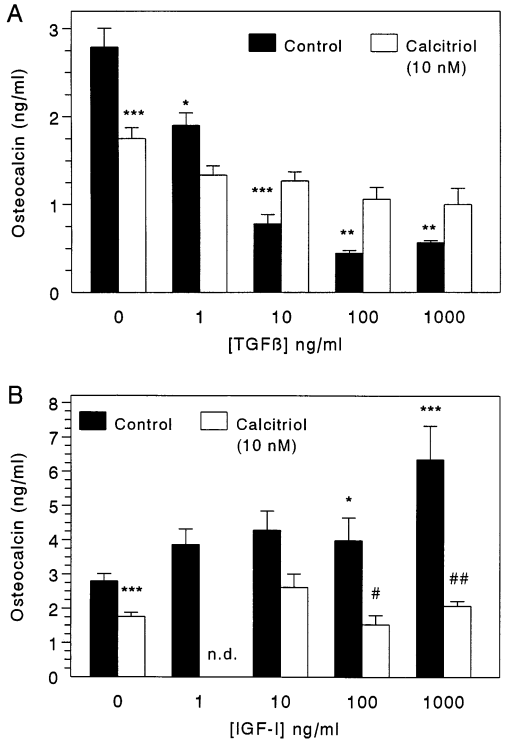

FIG. 3. The effects of TGF-β and IGF-I in the absence or presence of $1,25(OH)_2D_3$ on osteocalcin level in the medium of fetal mouse long bones. Fetal mouse long bones were incubated with various concentrations of (A) TGF-β and (B) IGF-I alone or in combination with 10^{-8} M $1,25(OH)_2D_3$ for 6 days. Osteocalcin level was measured after 6 days. Data are presented as mean ± SEM of 4-20 cultures. n.d., not determined; $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ vs. control. $\#p < 0.05$, $\#\#p < 0.005$ calculated as significance of interaction.

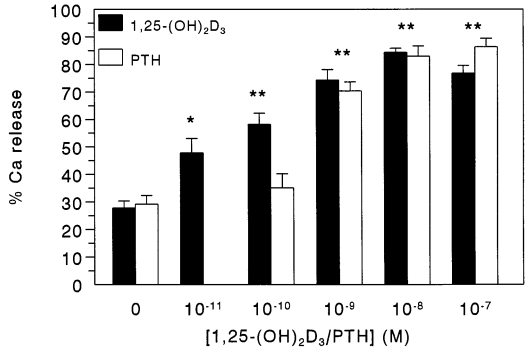

FIG. 4. The effects of the systemic factors $1,25(OH)_2D_3$ and PTH on bone resorption in fetal mouse long bones. Prelabeled fetal mouse radii/ulnae were incubated with the indicated concentrations of $1,25(OH)_2D_3$ or PTH for 6 days, and the release of ^{45}Ca into the medium was determined. Percent ^{45}Ca release is a measure for bone resorption and was assessed as described in Materials and Methods. Data are presented as mean ± SD of 12 cultures. $^*p < 0.005$, $^{**}p < 0.0001$ vs. control.

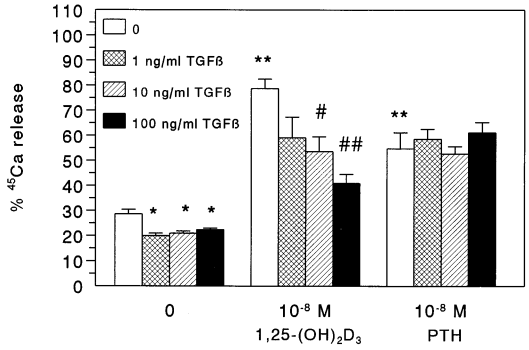

FIG. 5. The effects of TGF-β on $1,25(OH)_2D_3$- and PTH-stimulated bone resorption in fetal mouse long bones. Prelabeled radii/ulnae were incubated with several concentrations of TGF-β (0-100 ng/ml) in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$, or 10^{-8} M PTH for 6 days (two times 3 days). Data obtained after 6 days are presented. After 3 days, similar but less-pronounced effects were observed. Data are presented as mean ± SEM of 12 cultures. $^*p < 0.01$, $^{**}p < 0.0001$ vs. control. $\#p < 0.05$, $\#\#p < 0.0001$ calculated as significance of interaction.

bone resorption (Fig. 6). However, the PTH-stimulated bone resorption is about 10 times less sensitive to IGF-I than the $1,25(OH)_2D_3$-stimulated bone resorption.

Analysis of osteocalcin in relation to bone resorption

Although serum osteocalcin level is clinically used as a marker of bone turnover, its precise function is still unknown. Osteocalcin has been postulated to play a role in the control of mineralization but also to have a function in bone resorption. Osteocalcin has been suggested to play a role in bone resorption by enhancing the recruitment and differentiation of osteoclast precursors.[31,32] In the present study, we had the opportunity to examine directly the relationship between stimulation of bone resorption and production of osteocalcin because in the same long bones in which bone resorption was studied the osteocalcin response was measured. A negative correlation is suggested by the above presented opposite effects of $1,25(OH)_2D_3$ on bone resorption and osteocalcin production. In Fig. 7, the regression analysis of these data are presented. This analysis demonstrates that the extent of bone resorption by $1,25(OH)_2D_3$ is significantly and inversely related to osteocalcin production. Although less pronounced than in the $1,25(OH)_2D_3$- stimulated stituation, basal osteocalcin production and bone resorption are also inversely related both after 3 days (not significant) and 6 days ($p < 0.025$) of culture (data not shown).

DISCUSSION

The data obtained in the present study support the hypothesis that bone-derived growth factors interact with the

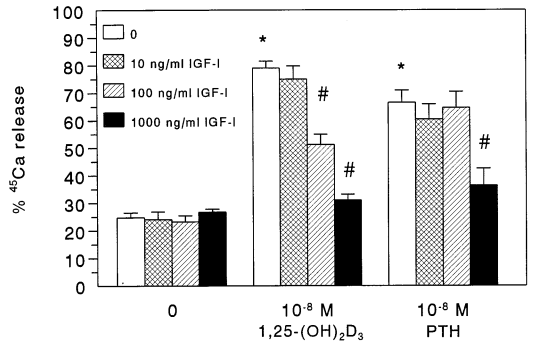

FIG. 6. The effects of IGF-I on $1,25(OH)_2D_3$- and PTH-stimulated bone resorption in fetal mouse long bones. Prelabeled radii/ulnae were incubated with several concentrations of IGF-I (0-1000 ng/ml) in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$ or 10^{-8} M PTH for 6 days (two times 3 days). Data obtained after 6 days are presented. After 3 days of incubation, similar but less pronounced effects were detected. Data are presented as mean ± SEM of 12 cultures. $^*p < 0.0001$ vs. control. $\#p < 0.005$, $\#\#p < 0.0001$ calculated as significance of interaction.

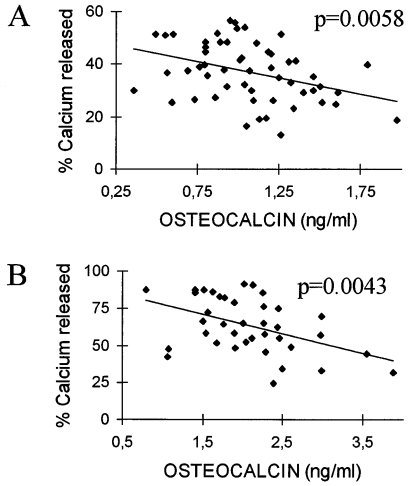

FIG. 7. Regression analysis of bone resorption and osteocalcin in $1,25(OH)_2D_3$-treated fetal mouse long bone cultures. ^{45}Ca prelabeled radii/ulnae were incubated two times for 3 days with various concentrations of $1,25(OH)_2D_3$. After (A) 3 and (B) 6 days of culture in the same medium, the amount of osteocalcin and percentage of released ^{45}Ca were determined. Next, using linear regression analysis, the correlation between osteocalcin and bone resorption was assessed.

systemic regulators of bone metabolism, $1,25(OH)_2D_3$ and PTH, in the control of bone turnover as assessed by the regulation of bone resorption and osteocalcin production. In previous studies, we demonstrated that TGF-β potently blocks $1,25(OH)_2D_3$-induced osteocalcin expression in rat and human osteoblastic cells via interference at the transcription level.[24,26] Initially, surprisingly we found that in fetal mouse long bones, $1,25(OH)_2D_3$ dose-dependently reduced osteocalcin synthesis. Analysis of bone extracts showed that this decrease of osteocalcin in the medium was not the result of accumulation in the bone tissue. The decrease was also not specific for the fetal system because with the neonatal MC3T3 mouse osteoblast-like cells inhibition of osteocalcin synthesis was also found. After completing our study, data were presented that demonstrated inhibition of osteocalcin mRNA expression in mouse osteoblast-like cells by $1,25(OH)_2D_3$.[33,34] Previously, it was shown that the hypophosphatemic *Hyp* mice have increased serum osteocalcin levels, which are reduced by $1,25(OH)_2D_3$ treatment.[35] This was not due to altered affinity for hydroxyappatite or renal handling but is the result of an altered gene expression.[36] In contrast, in normal mice they observed an increase in serum osteocalcin and osteocalcin mRNA expression in calvaria after $1,25(OH)_2D_3$ treatment.[35,36]

The difference between rat/human and mouse indicates species specificity in the regulation of osteocalcin production by $1,25(OH)_2D_3$. This is supported by data of Sztajnkrycer et al.,[37] who reported that in ROS 17/2.8 cells stably transfected with the mouse osteocalcin genes, $1,25(OH)_2D_3$ also reduced mouse osteocalcin production, whereas the endogenous rat osteocalcin gene was stimulated by $1,25(OH)_2D_3$. Also, in mouse MC3T3-E1 osteoblasts stably transfected with a rat osteocalcin promoter-CAT reporter gene, the endogenous mouse osteocalcin production is reduced while CAT activity is increased by $1,25(OH)_2D_3$.[33] Kesterson et al.[38] introduced the human osteocalcin gene promoter fused to the chloramphenicol acetyltransferase gene into the germ line of mice and showed reduction of CAT activity after a low vitamin D_3 diet, while $1,25(OH)_2D_3$ supplementation restored and enhanced CAT activity over control values. These data show that the differences observed between rat/human and mouse are not due to differences in the availability of factors important for the regulation of the osteocalcin gene. Remarkable is the difference in the 5′ half site of the vitamin D responsive elements (VDREs) between the rat (GGGTGAnnnAGGACA),[39–41] human (GGGT GAnnnGGGGCA),[42,43] and mouse (GGGCAAnnnAG GACA)[44] VDREs of the osteocalcin genes (underlined are the nucleotides in the mouse VDRE that differ from both the rat and human genes). This dinucleotide difference in the VDRE may lead to a change in its ability to bind nuclear proteins, and thus, eventually, in a change in regulation of transcription. However, a recent study by Zhang et al. indicated an indirect effect of $1,25(OH)_2D_3$ on mouse osteocalcin genes by abolishing binding of an osteoblast-specific transcription factor.[34]

TGF-β reduced the basal osteocalcin level in the fetal mouse long bone cultures. This inhibition is in line with observations with rat and human osteoblast-like cells[24,26,30,45]; however, the effect in the mouse long bones was much more pronounced. The inhibition by TGF-β was more potent than the inhibition by $1,25(OH)_2D_3$. No significant interaction between TGF-β and $1,25(OH)_2D_3$ was observed. TGF-β and $1,25(OH)_2D_3$ together did not result in a synergistic stronger inhibition or an additive effect. In contrast, in the presence of $1,25(OH)_2D_3$, the osteocalcin level was higher than that of TGF-β alone. The background of this observation is unclear and yet purely speculative. Together,

the present data obtained with fetal mouse long bone do not demonstrate a strong interaction between these two compounds as it has been demonstrated in rat and human osteoblastic cells. Besides TGF-β, the effects of IGF-I were examined. In the mouse long bones, IGF-I increased the basal osteocalcin level, which is in line with data obtained with osteoblastic cells of human origin and rat calvaria and in vivo human studies, showing that an increase in serum IGF-I levels is followed by an increase in serum osteocalcin levels.[46-49] In contrast to TGF-β, with IGF-I, a significant interaction with $1,25(OH)_2D_3$ was observed. Taking these osteocalcin data together, it can be concluded that these bone-derived growth factors each have a specific and different role in the control of osteocalcin production either alone or in combination with $1,25(OH)_2D_3$. This thereby demonstrates that control of osteocalcin during bone turnover is dependent on the presence or absence of particular growth factors in combination with $1,25(OH)_2D_3$.

In the same fetal mouse long bone cultures used for osteocalcin analyses, interaction between these growth factors and $1,25(OH)_2D_3$ with respect to bone resorption was examined. Also for bone resorption, growth factor–specific interactions with $1,25(OH)_2D_3$ were detected. $1,25(OH)_2D_3$-stimulated bone resorption is regulated in a growth factor–specific manner. Moreover, the effects of TGF-β and IGF-I are hormone specific. PTH-stimulated bone resorption is differently affected by these growth factors than $1,25(OH)_2D_3$-stimulated bone resorption. We observed that TGF-β inhibits $1,25(OH)_2D_3$- but not PTH-stimulated bone resorption in fetal mouse long bones. On the contrary, IGF-I inhibited both $1,25(OH)_2D_3$- and PTH-stimulated bone resorption, although with different sensitivities. These data demonstrate that growth factors can play an important specific role in the control of the extent of bone resorption in response to $1,25(OH)_2D_3$ and/or PTH.

These growth factors are produced by bone cells and incorporated into the bone matrix. Preliminary immunohistochemical analyses show that these growth factors are not evenly distributed in the bone matrix but are restricted to certain areas (our unpublished data). This would implicate that these factors define the action of systemic factors at specific sites and may thereby be important in the regulation of the architecture of bone. For example, $1,25(OH)_2D_3$-induced bone resorption at a TGF-β–positive site may be limited whereas at a TGF-β–negative site resorption can go on. The proposed model requires that growth factors should be released from the bone matrix during resorption and must still be biologically active or in a configuration that can be activated. TGF-β is stored in a latent form in the bone matrix,[50,51] and IGF-I binds to IGFBPs, which are capable of binding the extracellular matrix.[18,52,53] These features can have protective effects for the growth factor to degradation and may allow the eventual release of active growth factors. This is supported by the in vitro observation that several factors that stimulate bone resorption at the same time enhance TGF-β activity in the culture medium.[54] This indeed suggests that TGF-β, but possibly also the other growth factors, may serve as a local feedback regulatory mechanism for bone resorption stimulated by calciotropic hormones. Possibly the recently demonstrated and discussed transcytosis of bone matrix proteins through osteoclasts can play an important role in this local feedback loop.[55-57]

The present results about differential regulation of $1,25(OH)_2D_3$ and PTH stimulation of bone resorption by the growth factors tested point to different bone resorptive mechanisms for $1,25(OH)_2D_3$ and PTH. $1,25(OH)_2D_3$, unlike PTH, has previously been shown to affect differentiation of precursors of the osteoclast lineage[58-60] and may thereby regulate bone resorption. Our data on the inhibitory effect of TGF-β on $1,25(OH)_2D_3$ but not PTH stimulation of bone resorption seems to be in line with these observations because TGF-β is a potent inhibitor of $1,25(OH)_2D_3$-induced osteoclast-like cell formation in long-term human bone marrow cultures, both by inhibiting proliferation and differentiation of early precursors and by inhibiting fusion of mononuclear cells to form osteoclasts.[61] In addition, in fetal rat long bones, TGF-β inhibition of $1,25(OH)_2D_3$-stimulated bone resorption was suggested to occur via the inhibition of osteoclast precursor proliferation.[62]

Despite the fact that osteocalcin is clinically used as a bone turnover marker, its function is still unclear. Among others, osteocalcin has been postulated to play a stimulatory role in bone resorption by enhancing the recruitment and/or differentiation of osteoclasts.[31,32] As shown by the regression analysis in Fig. 7, our data do not support a major role for osteocalcin in the $1,25(OH)_2D_3$ regulation of bone resorption in fetal mouse long bones. Although it is anticipated that in other species there is a positive correlation between osteocalcin production and stimulation of bone resorption because, e.g., in rat both responses are stimulated by $1,25(OH)_2D_3$, the current results show that in mouse long bones $1,25(OH)_2D_3$ can stimulate bone resorption independent of osteocalcin. However, the previous studies on a positive role of osteocalcin in the process of bone resorption should be taken into account.[31,32] The present data do not completely preclude a role for osteocalcin in the stimulation of bone resorption. Osteocalcin could still be involved in the recruitment and differentiation of osteoclasts, a process which is less prominent in the fetal radii/ulnae system used in the present study. Moreover, we measured a decrease in the medium of the long bones while matrix-bound osteocalcin did not significantly change, and it may be that the bound fraction is more important for bone resorption than the soluble fraction. However, our present observations seem to be in line with the recently published data on the osteocalcin-deficient mice, which demonstrated an increase in bone formation without affecting bone resorption.[63]

In conclusion, the data on synthesis of these growth factors by osteoblasts, their presence in bone matrix, their effects on bone cells, and their interactions with systemic regulators of bone metabolism at osteoblast level has led to the hypothesis that they play an important role in bone metabolism. The present study supports this concept by showing growth factor–specific interactions between TGF-β, IGF-I, and $1,25(OH)_2D_3$ with respect to bone resorption and osteocalcin production. Studies with PTH also demonstrated hormone-specific effects of these growth fac-

tors regarding bone resorption. The mechanism by which $1,25(OH)_2D_3$- and PTH-enhanced bone resorption are specifically regulated by TGF-β and IGF-I has still to be elucidated. However, the present study provides a basis for a local regulatory mechanism of bone resorption stimulated by $1,25(OH)_2D_3$ and PTH.

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