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Differential Regulation of Cbfa1/Runx2 and Osteocalcin Gene Expression by Vitamin-D3, Dexamethasone, and Local Growth Factors in Primary Human Osteoblasts

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Abstract Core binding factor alpha 1 (Cbfa1) is an osteoblast-specific transcription factor essential to develop a mature osteoblast phenotype. However, its exact role in the signaling of various osteotropic-differentiating agents is still unclear. In this study, we assessed the effects of 1,25-$(OH)_2$-D3 (D3), ascorbic acid, bone morphogenetic protein-2 (BMP-2), dexamethasone (Dex), and transforming growth factor-β (TGF-β) on Cbfa1 and osteocalcin (OCN) mRNA steady state levels (by semiquantitative RT-PCR) in an in vitro model of osteoblast differentiation. TGF-β increased Cbfa1 mRNA levels in normal primary human osteoblasts (pHOB) by 2.6-fold in a time-dependent fashion with maximum effect on day 28 ($P < 0.001$). Similarly, the glucocorticoid Dex enhanced Cbfa1 gene expression by pHOB in a time-dependent fashion by up to 4.6-fold ($P < 0.001$). In contrast, Dex inhibited OCN gene expression levels by 68% ($P < 0.01$). Treatment with BMP-2 resulted in an earlier enhancement of Cbfa1 and led to a 4.2-fold increase with a maximum on day 21 ($P < 0.001$). Ascorbic acid did not modulate Cbfa1 and OCN gene expression. The effect of vitamin D (D3) on Cbfa1 mRNA expression was influenced by the duration of treatment, being inhibitory after 1 h and having a stimulatory effect after 48 h. Time course experiments indicated a stimulatory effect of D3 on Cbfa1 mRNA levels (by 2.5-fold after 48 h; $P < 0.01$). Analysis of the late cellular differentiation marker osteocalcin revealed that D3 increased OCN gene expression by 14-fold ($P < 0.001$). In conclusion, in normal primary human osteoblasts, the rapid and pronounced increase of OCN after treatment with D3 seems not to be mediated by Cbfa1. These data imply that Cbfa1 gene expression is differentially regulated by various osteoblastic differentiating agents and is dependent on the stage of maturation. J. Cell. Biochem. 86: 348–356, 2002.

Key words: BMP-2; Cbfa1; differentiation; glucocorticoids; osteoblast; osteocalcin; vitamin D; TGF-β

Bone formation is a complex process involving vitamins, hormones, and local growth factors. The central operator is the osteoblast that passes through several maturation stages in an ordered time sequence, developing into the fully active phenotype. Osteoblasts are derived from mesenchymal stem cells, which have the potential to differentiate into chondrocytes, myocytes, and adipocytes. The runt family transcription factor (Cbfa1/Runx2) is a recently described osteoblast-specific transcription factor which is essential for the differentiation process of mesenchymal stem cells into mature osteoblasts and for maintaining the differentiated function in these cells during bone formation and remodeling [Merriman et al., 1995; Mundlos et al., 1997]. Cbfa1-deficient mice showed a complete lack of bone formation and immature osteoblast with missing osteopontin and osteocalcin (OCN) production underlining the essential role of this factor for osteoblastogenesis and osteoblast function [Komori et al., 1997]. Although OCN is not essential for mineralization in OCN-deficient mice [Ducy

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et al., 1996], it is highly expressed during the last stage of bone formation. An influence of Cbfa1 on OCN expression can be expected in view of several Cbfa1 binding sites in the OCN gene promotor [Ducy and Karsenty 1995; Frendo et al., 1998].

How Cbfa1 is integrated into the multifactorial network of bone differentiation is still unclear. Species differences have been shown and data on human cells are restricted to tumor cell lines or immortalized cells [Prince et al., 2000]. In particular, no data exists on Cbfa1 gene expression in long-term cultures of pHOB. In previous studies, we have shown that pHOB cells display a time-dependent pattern of gene expression and protein secretion during differentiation in culture [Siggelkow et al., 1999a] in part similar to rat calvaria osteoblasts as described by Owen et al. [1990]. Therefore, we investigated in pHOB cells whether Cbfa1 is a major target gene for dexamethasone (Dex), 1,25-$(OH)_2$-D3 (D3) and local growth factors, known to modulate bone differentiation and mineralization as well as the regulatory function of the osteoblasts. Furthermore, we were interested whether 4Cbfa1 gene induction was correlated to OCN gene expression, a characteristic component of the bone matrix produced by the highly differentiated osteoblast.

MATERIALS AND METHODS

Materials

Cell culture medium and supplements were purchased from Invitrogen (Karlsruhe, Germany). Culture flasks and dishes were obtained from Nunc (Roskilde, Denmark). Recombinant human bone morphogenetic protein-2 (rhBMP-2) was a generous gift from Dr. J Clement, Children's Hospital, Jena, Germany, human transforming growth factor-β (TGF-β) was obtained from PromoCell, (Heidelberg Germany), Dex and ascorbic acid from Sigma (Munich, Germany). Unless stated otherwise, all other chemicals were purchased from Sigma.

Cell Culture

Bone specimens were obtained from the iliac crest of five patients (four women, one man; age 36.2 ± 5.2 years) undergoing corrective surgery after traumatic fracture. None of the patients had signs or symptoms of bone or autoimmune diseases. The study was approved by the Institutional Review Board of the University of Goettingen. Following written informed consent, we obtained RNA from primary second-passage osteoblastic cells (pHOB) from cultures of trabecular bone explants as previously described [Beresford et al., 1986; Siggelkow et al., 1999b]. These pHOB cells were grown at 37°C and maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) from Biochrom (Berlin, Germany). Cell confluence was reached on day 10. Unless stated otherwise, standard concentrations of differentiating agents for a constant exposure time of 24 h were used [Beresford et al., 1984; Auf'm Kolk et al., 1985; Siggelkow et al., 1999b].

RT-PCR Analysis

Total cellular RNA was isolated using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). Reverse transcription was performed with 1 μg of total RNA as previously described [Viereck et al., 2002a]. Each cDNA sample was run in triplicate for each PCR reaction. Competitive RT-PCR was performed using exogenous DNA competitors ("mimics") as internal control [Viereck et al., 2002a] that were synthesized with the PCR mimic construction kit from Clontech (Palo Alto, CA). PCR reactions were carried out in 15 μl reactions using primer sequences as previously described [Rickard et al., 1996; Viereck et al., 2002a] and cycle numbers ensuring a linear amplification profile. The ribosomal house-keeping gene L7 and OCN mRNA were analyzed as reported elsewhere [Rickard et al., 1996; Viereck et al., 2002b], core binding factor alpha 1(Cbfa1) mRNA was analyzed using a protocol of 2 min at 95°C, 30 cycles (of 30 s at 95°C, 30 s at 59°C, 1 min at 72°C), 10 min at 72°C. The oligonucleotides for Cbfa1 (sense: 5′-CCACCTCTGACTTCT GCCTC-3′; antisense: 5′-GACTGGCGGGGTGT AAGTAA-3′) (GenBank Accession #AH 005498) were synthesized at MWG-Biotech (Ebersberg, Germany). Target and mimic sizes were 378 and 548 bp (L7), 172 and 340 bp (Cbfa1), and 310 and 438 bp (OCN). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. The expression of each gene was quantified as target to mimic ratio and normalized to L7. To ensure specificity of the PCR products, the amplification product was sequenced with the Abi Prism system from Perkin Elmer (Weiterstadt, Germany).

Statistical Analysis

Unless stated otherwise, all experiments were reproduced at least three times. Values are expressed as the mean ± SEM of triplicate measurements and data obtained from representative experiments are shown. For analysis of time course experiments and evaluation of differences between the sample of interest and its respective control, multiple-measurement ANOVA followed by Newman–Keuls post-test analysis was used if not noted otherwise. A P value of less than 0.05 was considered statistically significant.

RESULTS

The pHOB cells displayed a characteristic pattern of gene expression and protein production of various osteoblastic differentiation markers (Type I collagen, alkaline phosphatase, and osteocalcin) and were developmentally regulated over time in culture [Siggelkow et al., 1999a and unpublished data]. Under basal conditions, Cbfa1 gene expression significantly increased in the second half of the culture period with a maximum at day 21 ($P < 0.001$). In contrast, OCN gene expression in control cultures did not change significantly with time, indicating that pHOB cells reach the matrix maturation stage but not the mineralization stage when cultured under basal conditions only [Siggelkow et al., 1999a]. However, under appropriate conditions, the pHOB cells represent the mature osteoblastic phenotype and matrix calcification was reached [Siggelkow et al., 2002]. In all experiments, no gender differences could be observed.

To assess the effects of various differentiating agents, such as TGF-β, BMP-2, ascorbic acid, Dex, and vitamin D (D3), on Cbfa1 and OCN mRNA expression, we treated second-passage pHOB cells either with vehicle (ethanol) or agent in time course experiments. TGF-β for 24 h increased Cbfa1 mRNA steady state levels (as assessed by semiquantitative RT-PCR) in a time-dependent manner ($P < 0.0001$ by ANOVA; Figs. 1 and 2A). TGF-β increased Cbfa1 mRNA levels at a concentration of 1 ng/ml by 2.6-fold with the maximum effect on day 28 ($P < 0.001$). In contrast, OCN gene expression was not affected by TGF-β at any time point investigated (Fig. 2B).

BMP-2 at a concentration of 50 ng/ml for 24 h resulted in a faster and more pronounced

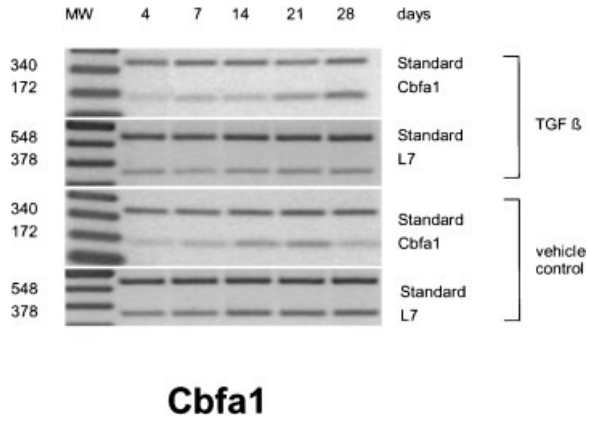

Fig. 1. TGF-β stimulates Cbfa1 mRNA levels in a time-dependent manner. Semi-quantitative RT-PCR analysis of Cbfa1 mRNA levels from pHOB that were cultured for the time indicated (in days) in the presence of TGF-β (1 ng/ml for the last 24 h) or vehicle control (ethanol). The results are representative of five independent experiments. The expression of Cbfa1 was quantified as target to standard (mimic) ratio and normalized to L7 and is shown in Figure 2A.

enhancement of the transcription factor Cbfa1 as compared to TGF-β. Specifically, BMP-2 led to an initial 4.2-fold increase of Cbfa1 mRNA after 7 days with maximum on day 21 and a subsequent decrease ($P < 0.0001$ by ANOVA; Fig. 2C). For OCN gene expression, no significant differences between stimulated and control group could be observed except on day 7, when a small but significant decrease was detectable ($P < 0.01$, Fig. 2D).

In order to characterize the effects of Dex on Cbfa1 and OCN gene expression, pHOB cells were treated with Dex 10^{-8} M for 24 h. Dex produced considerable effects on both parameters. Dex induced Cbfa1 mRNA steady state levels up to 4.6-fold, with maximum values on day 28 ($P < 0.001$, Fig. 2E). In contrast and as expected from clinical observations, Dex inhibited OCN gene expression by 68% ($P < 0.01$) (Fig. 2F).

Treatment with ascorbic acid at a concentration of 50 μg/ml increased Cbfa1 mRNA levels only weakly (by 29%; $P > 0.05$) and had no effects on OCN gene expression (data not shown).

To determine whether the stimulatory effects of vitamin D (D3) on OCN production in osteoblastic cells are also mediated by the transcription factor Cbfa1, time courses were performed. For time course experiments, the pHOB cells were treated at confluence with D3 at a concentration of 5×10^{-8} M for 0, 1, 4, 12, 24, and 48 h. Treatment with D3 increased Cbfa1 mRNA levels only weakly (by 67% after 48 h;

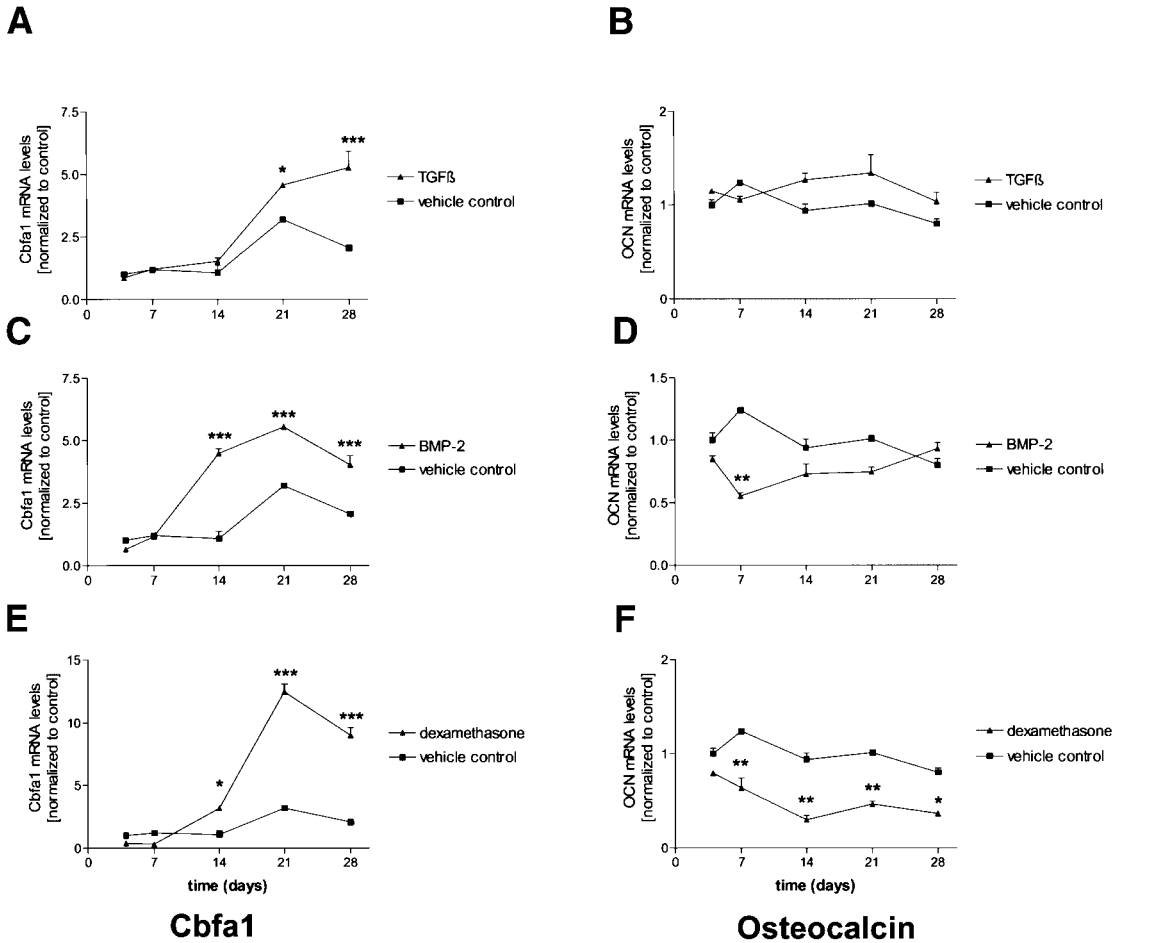

Fig. 2. Semi-quantitative RT-PCR analysis for Cbfa1 (**left**) and osteocalcin (**right**) gene expression by pHOB cells over time (in days) in culture. RT-PCR demonstrating regulation of Cbfa1 and OCN gene expression by TGF-β (1 ng/ml), rhBMP-2 (50 ng/ml), dexamethasone (Dex, 10^{-8} M), or vehicle control (ethanol) for 24 h at the days indicated. Cell confluence was reached on day 10. Data represent the mean ± SEM of triplicates of target gene/L7 ratios normalized to control on day 4 (percent of control). $P < 0.0001$ by ANOVA for (**A**), (**C**), (**E**), and (**F**); Post-test Newman–Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for individual values compared to respective controls.

$P < 0.05$; data not shown). Of note, Cbfa1 mRNA levels were decreased by 38% ($P < 0.05$; data not shown), whereas no effect on OCN gene expression was seen after treatment with D3 for 1 h.

Next, we analyzed the differential regulation of Cbfa1 and OCN gene expression after short (1 h) or long-term (48 h) incubation with D3 in time course experiments for up to 32 days (Fig. 3). Compared to vehicle treatment, D3 markedly decreased Cbfa1 mRNA expression ($P < 0.0001$ by ANOVA; Fig. 3A) with maximal inhibitory effects of 59% on day 24. Together, these results indicate that D3 for 1 h inhibits Cbfa1 gene expression at the transcriptional level. OCN gene expression was not influenced by 1 h treatment with D3 (Fig. 3B). In order to further characterize the effects of vitamin D on Cbfa1 gene expression, the pHOB cells were treated with either vehicle and D3 (5×10^{-8} M for the last 48 h prior to RNA isolation). D3 increased Cbfa1 mRNA steady state levels by pHOB in a time-dependent fashion by 2.5-fold on day 14 ($P < 0.01$; Fig. 3C). OCN mRNA was highly stimulated at all time points with maximum at day 14 by 14-fold ($P < 0.001$; Fig. 3D).

DISCUSSION

In this study, we investigated whether Cbfa1 gene expression in human primary osteoblasts (pHOB) is differentially regulated during time in culture. Cbfa1 plays a key role in both, the commitment and the development of skeletal lineage cells responsible for bone tissue.

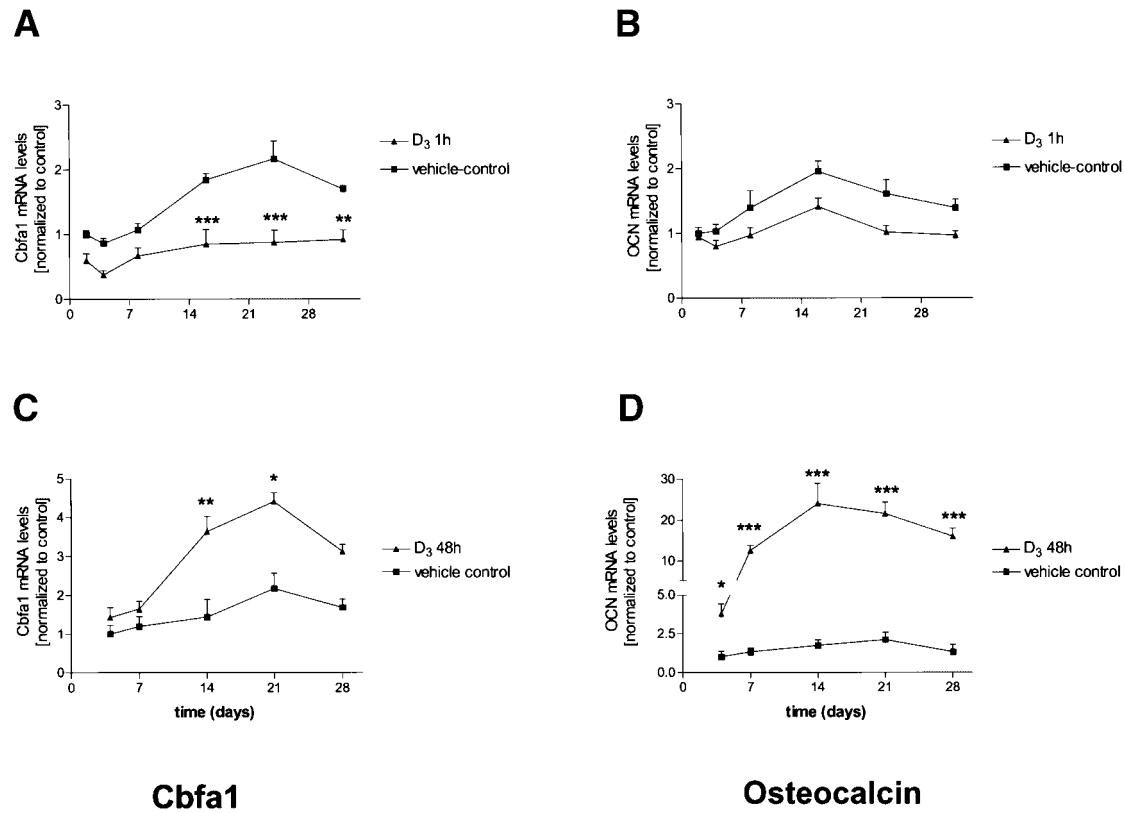

Fig. 3. Semi-quantitative RT-PCR analysis for Cbfa1 (**left**) and osteocalcin (**right**) gene expression by pHOB cells over time (in days) in culture. RT-PCR demonstrating regulation of Cbfa1 and OCN gene expression after 1 h (**upper panel**) or 48 h (**lower panel**) treatment with 5×10^{-8} M vitamin D (D3) or vehicle control (ethanol) at the days indicated. Cell confluence was reached on day 10. Data represent the mean ± SEM of triplicates of target gene/L7 ratios normalized to control on day 4 (percent of control). $P < 0.0001$ by ANOVA for (**A**); Post-test Newman–Keuls analysis: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for individual values compared to respective controls.

Because cytokines, growth factors, and steroid hormones significantly influence proliferation and differentiation properties of bone cells, we addressed Cbfa1 as a target gene for regulation by TGF-β, BMP-2, Dex, and D3. Although Cbfa1 has been studied in vitro using rodent cells and cell lines, it has to be questioned whether results can be transferred to normal human cells [Chang et al., 1998; Prince et al., 2000].

In accordance with the general assumption that Cbfa1 expression increases with differentiation [Chang et al., 1998], maximal Cbfa1 mRNA levels were measurable after 3 weeks of culture under basal conditions. The stimulatory effects of TGF-β, BMP-2, and Dex added to the differentiation-dependent behavior of Cbfa1 gene expression. BMP-2 and Dex had no influence on Cbfa1 in the proliferative phase (day 4–7) but stimulated Cbfa1 gene expression after confluence, indicating a differential regulation of Cbfa1 gene expression in pHOB cells during maturation in culture. Starting at day 21, TGF-β increased Cbfa1 mRNA levels. These data are in contrast to recent results in three immortalized human osteoblast cell lines representing three different stages of differentiation showing unchanged mRNA levels during time in culture and no influence of TGF-β or D3 [Prince et al., 2000].

TGF-β seems to act as a bifunctional regulator of cell growth, stimulating the transition from proliferation to matrix synthesis, but inhibiting mineralization [Bonewald et al., 1992; Breen et al., 1994]. In C2C12 myogenic cells, TGF-β treatment induced Cbfa1 expression but was insufficient to support osteoblast differentiation reflected by the absence of alkaline phosphatase, osteopontin, and OCN [Lee et al., 1999].

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-superfamily. BMP-2 initiates the differentiation of multipotent mesenchymal progenitor cell lines to the osteogenic lineage, as assessed

by induction of alkaline phosphatase activity and expression of osteoblast markers, such as OCN [Katagiri et al., 1990; Yamaguchi et al., 1991; Thies et al., 1992; Ahrens et al., 1993]. Furthermore, BMPs are able to promote the differentiation of osteoblasts in vitro and to induce bone formation in several cell culture systems [Wang et al., 1990; Sampath et al., 1992; Thies et al., 1992]. It is unknown, however, whether BMPs may promote also the human osteoblastic phenotype by increasing the growth or differentiation of cells at different stages of maturation. An increase of Cbfa1 expression by BMP-2 has been consistently demonstrated in immortalized human neonatal calvarial cells [Hay et al., 2000], in an immortalized human marrow stromal cell line (hMS (2–6)) [Gori et al., 1999], in mouse pluripotent mesenchymal precursor cells (C2C12) [Lee et al., 1999], and in a chondrocyte like cell line (TC6) [Takazawa et al., 2000]. In a conditionally immortalized human marrow stromal cell line (hMS (2–6)), BMP-2 enhanced osteoblast differentiation as assessed by a 1.8-fold increase in expression of Cbfa1. In addition, Cbfa1 was expressed as the initial change in the BMP-2 signaling cascade regulating osteoblast differentiation [Gori et al., 1999]. In our experiments in pHOB cells, BMP-2 started to stimulate Cbfa1 mRNA levels by 4.2-fold at the beginning of the maturation phase. The present data support the assumption that TGF-β and BMP-2 stimulate osteoblast differentiation and that Cbfa1 might be a common signaling pathway [Lee et al., 1999]. In this context, findings of Bae et al. [2001] in mouse C2C12 cells are of interest showing that TGF-β and BMP-2 stimulated Cbfa1 expression, inhibited their differentiation into myotubes (TGF-β) and initiated osteoblastic differentiation (BMP-2). Therefore, both factors may increase Cbfa1 resulting in different functions during osteogenesis.

The effects of Dex were very similar to those of TGF-β and BMP-2. Again a very pronounced increase of Cbfa1 gene expression in the second half of the culture period was observed. This is in accordance to the widely accepted idea that Dex is a strong differentiation factor for osteoblasts [Shalhoub et al., 1992; Cheng et al., 1994]. Recently, Prince et al. [2000] reported that Dex induced an increase of Cbfa1 protein but not of mRNA in conditionally immortalized human osteoblast cell lines. They postulated multiple levels of regulation of Cbfa1 expression and activity. Their findings were in contrast to modifications in Cbfa1 expression in primary rat osteoblasts and in a mouse MC3T3 cells [Prince et al., 2000].

OCN is a bone-specific matrix protein characteristic for the late phase of osteoblast differentiation and considered essential for calcification of the bone matrix. However, in OCN-deficient mice, mineralization was even increased [Ducy et al., 1996] attributing to OCN a more inhibiting role during mineralization. It seemed suggestive that the regulation of OCN expression might be closely linked to that of Cbfa1 because several Cbfa1 binding sites are located in the OCN promotor. In a myoblastic cell line (C2C12), induction of collagen Type I and alkaline phosphatase (AP) expression after BMP-2 treatment coincided with Cbfa1 expression, while Cbfa1 mRNA was strikingly down regulated at the onset of expression of OCN gene [Lee et al., 1999] showing a negative correlation between Cbfa1 and OCN. Lee and co-workers were able to show in myogenic C2C12 that maximal suppression of OCN promoter activity in response to BMP-2 requires sequences in the proximal promoter and may occur independent of the three Cbfa binding sites [Lee et al., 1999]. The significance of Cbfa1 for BMP-2 signaling in relation to OCN expression was also questioned by experiments in embryonic osteoblasts of Cbfa1-deficient mice [Komori et al., 1997]. It was possible to increase OCN production by BMP-2, which suggests that other transcription factors than Cbfa1 are involved.

In pHOB cells, TGF-β had no effect on OCN mRNA levels, whereas BMP-2 showed an inhibiting effect on OCN mRNA in the proliferative phase, reaching control levels after 28 days of culture. This is in contrast to the BMP-2 effect on Cbfa1 gene expression with no effect during proliferation but stimulation during osteoblast maturation beginning after confluence. A similar behavior of OCN gene expression, when stimulated by BMP-2, was reported by Gori et al., [1999] in human marrow stromal cells (hMS (2–6)) when a short increase of OCN mRNA expression during continuous BMP-2 stimulation could be observed after 4 days with normalization at day 8.

The influence of Dex on Cbfa1 and OCN gene expression is controversial. Dex has a catabolic effect on the skeleton leading to osteoporosis. Therefore, an effect on Cbfa1 and/or OCN expression is very suggestive. We found a strong

increase in Cbfa1 expression but a suppression of OCN mRNA, the latter being expected because a decrease of plasma OCN during glucocorticoid treatment is well known. In contrast in rat calvarial cells, Dex stimulated OCN mRNA levels beginning after confluence [Shalhoub et al., 1992]. The effect of Dex on differentiating osteoblasts is also dependent on the duration of Dex treatment, short- and long-term effects may differ [Shalhoub et al., 1992].

Vitamin D3 is a potent differentiation factor for osteoblasts, inducing the transition from proliferation to differentiation [Owen et al., 1991]. Data on the effect of D3 on Cbfa1 expression are very limited. Interestingly, 1-h treatment decreased Cbfa1 mRNA independent of the stage of differentiation, whereas 48-h treatment showed a stimulatory effect. The biological significance of this effect is unclear at present. Thus, as with most early genes that code for transcription factors, a regulation of Cbfa1 expression during D3 stimulation occurred very early after treatment (1 h) as transient suppression, well before the increase of OCN mRNA was detectable. After 4-h incubation with D3, a steep increase of OCN mRNA was seen but no significant change of Cbfa1 gene expression. Therefore, the well-known stimulatory effect of D3 on OCN gene expression (and protein production) seems not to be mediated directly by Cbfa1. The increase of Cbfa1 after 48 h might reflect further differentiation of osteoblasts in culture.

Limitations of our study include cellular heterogeneity and donor-to-donor variability, features that are inherent to all studies employing patient-derived primary non-clonal osteoblastic cell models. In addition, we want to remind that gene expression at the mRNA level is not a final biological endpoint in regulating bone metabolism. Post-transcriptional regulation may be very important and may increase the activity of the gene product without changing mRNA expression. In this context, Xiao et al. [1998] reported a pronounced increase of Cbfa1 binding to its cognate DNA binding sequence by the influence of ascorbic acid, whereas Cbfa1 mRNA and protein levels were less affected. In our study, no effect of ascorbic acid on Cbfa1 gene expression levels was detectable.

In conclusion, our results indicate that Cbfa1 gene expression in pHOB cells increases after confluence and is further stimulated by various osteotropic agents. Given the findings on the gene expression level reported here, the OCN gene is not a major target of Cbfa1 when BMP-2, TGF-β, or Dex are the dominating stimulators. However, constitutive expression of Cbfa1 may be required to sustain the osteoblast phenotype and, probably in cooperation with other factors may initiate and modulate OCN gene expression. Our data also suggest that some factors, which modulate the differentiation and stimulate the expression of specific functions of the committed osteoblast-like cell, seem to bypass this key regulator. However, it is obvious that we are far from understanding this highly complex regulatory network, leading to a balanced and mechanical stress adapted bone metabolism and function.

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