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Transforming growth factor β-induced dissociation between vitamin D receptor level and 1,25-dihydroxyvitamin D_3 action in osteoblast-like cells†

A. Staal, J.C. Birkenhäger, H.A.P. Pols, C.J. Buurman, T. Vink-van Wijngaarden, W.M.C. Kleinekoort, G.J.C.M. van den Bemd, J.P.T.M. van Leeuwen*

Department of Internal Medicine III, Erasmus University Medical School, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

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Abstract

In the present study the interaction between a locally produced factor in bone, transforming growth factor β (TGFβ) and a systemic regulator of bone metabolism, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) was investigated. In rat (UMR 106, ROS 17/2.8) and human (MG-63) osteoblastic cell lines and in isolated fetal rat osteoblasts TGFβ caused a comparable increase in vitamin D receptor (VDR) level. A maximum was observed after 6 h at 1 ng/ml TGFβ. Scatchard analysis revealed that up-regulation of VDR is due to an increase in receptor number and not to a change in affinity. This was supported by Northern blot analysis which showed a dose- and time-dependent increase in VDR mRNA by TGFβ. To assess the significance of the TGFβ-induced increase in VDR level for 1,25-$(OH)_2D_3$ effects cells were preincubated with TGF for 4 h (causing a 2–3-fold increase of the VDR level) and subsequently incubated with 1,25-$(OH)_2D_3$ for 4 h and 24 h. TGFβ preincubation potently inhibited subsequent 1,25-$(OH)_2D_3$ stimulation of osteocalcin production in both ROS 17/2.8 and MG-63 cells on protein as well as mRNA level. A similar inhibition by TGFβ was observed on the 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA. The current study demonstrates dissociation between regulation of VDR level and modulation of two 1,25-$(OH)_2D_3$ biological responses by TGFβ in osteoblast-like cell lines of different origin. This dissociation shows that, besides interaction at VDR level also at other levels in the cell interaction(s) exist be-

* Corresponding author.

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tween TGFβ and 1,25-$(OH)_2D_3$. Besides, these data emphasize the potential importance of the interplay of locally produced factors and systemic calciotrophic hormones in the regulation of bone metabolism.

Keywords: TGFβ; 1,25-dihydroxyvitamin D_3; Vitamin D receptor; Osteocalcin; Osteopontin

1. Introduction

The seco-steroid 1,25-$(OH)_2D_3$ is an important regulator of calcium homeostasis. Besides stimulation of calcium absorption in the intestine, an important effect of 1,25-$(OH)_2D_3$ in response to a decrease in serum calcium concentration is stimulation of bone resorption [1]. The vitamin D receptor (VDR) in bone is located in the bone forming cell (osteoblast) and not in the bone resorbing cell (osteoclast) [2,3]. This indicates that 1,25-$(OH)_2D_3$ stimulates bone resorption indirectly via the osteoblast [4].

Regulation of receptor number is thought to be an important mechanism by which target cell responsiveness to hormones is modulated. Several factors, including 1,25-$(OH)_2D_3$ itself [5,6], glucocorticoids [7,8], parathyroid hormone [9,10], epidermal growth factor [11] and retinoic acid [12], have been demonstrated to modulate VDR level. Osteoblast responsiveness to 1,25-$(OH)_2D_3$ has been linked to VDR level. Induction of 24-hydroxylase activity by 1,25-$(OH)_2D_3$ closely paralleled VDR level after retinoic acid pretreatment [12]. For both a stimulatory (24-hydroxylase) and inhibitory (collagen synthesis) bioresponse [13] the magnitude of the response to 1,25-$(OH)_2D_3$ was directly correlated with the abundance of receptors (depending on rate of cell proliferation). Chen et al. [8,14] showed that receptor up-regulation increased the magnitude and sensitivity of responses to 1,25-$(OH)_2D_3$. However, with another steroid hormone, glucocorticoid, a dissociation was observed between receptor regulation and bioresponse [15].

Transforming growth factor β (TGFβ) is produced in bone, and present as a latent complex [16]. TGFβ has been thought to be activated during bone resorption by the low pH under the ruffled border of the osteoclast and to play an important role in the local regulation of bone remodeling [16,17]. TGFβ has been shown to increase VDR level in UMR 106-06 cells after 3 days of incubation [18]. Interaction between 1,25-$(OH)_2D_3$ and TGFβ has been described in MG-63 cells in the regulation of biological responses after coincubation [19].

In the present study we investigated the effect of TGFβ on VDR level in several osteoblast-like cell lines and studied the significance of this modulation of VDR level for 1,25-$(OH)_2D_3$ stimulation of osteocalcin production, osteocalcin mRNA and osteopontin mRNA synthesis. In general, this study emphasizes the importance of interactions between systemic and locally produced factors for the regulation of bone metabolism.

2. Materials and methods

2.1. Materials

We obtained [23,24-^{3}H]1,25-$(OH)_2D_3$ (105.5 Ci/mmol) from Amersham International (Aylesbury, Buckinghamshire, UK) and non-radioactive 1,25-$(OH)_2D_3$ was generously provided by LEO Pharmaceuticals BV (Weesp, The Netherlands). TGFβ type 2 was generously provided by Dr. J. Feyen, Sandoz Pharma Ltd (Basel, Switzerland). Fetal calf serum (FCS), αMEM medium, penicillin, streptomycin and glutamine were purchased from Flow Laboratories (Irvine, United Kingdom) and BSA fraction 5 from ICN ImmunoBiologicals (Lisle, USA). The rat VDR cDNA probe (1.7 kb) was generously provided by Dr. J.W. Pike (Houston, Texas), the rat osteocalcin probe (0.52 kb) and the rat osteopontin probe (1.3 kb) by Dr. M. Noda (West Point, Pennsylvania). Rat osteocalcin antiserum was a generous gift of Prof. R. Bouillon (Leuven, Belgium). All other reagents were of the best grade commercially available.

2.2. Cell culture

MG-63 cells were generously provided by Prof. R. Bouillon and ROS 17/2.8 cells by Dr. S.B. Rodan. UMR 106, ROS 17/2.8 (rat) and MG-63 (human) cells were seeded at 50 000 cells/cm^2 and cultured for 24 h with αMEM supplemented with 2 mM glutamine, 0.1% glucose, 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal calf serum (FCS). Next, medium was replaced by αMEM with 2% charcoal-treated FCS and cells were cultured for a 24-h or 48-h period during which the cells reached confluence. During this period, cells were incubated with the agents to be tested. Fetal rat osteoblasts were isolated from 20-day-old fetal calvaria by sequential EDTA and collagenase treatment [20], seeded at 25 000 cells/cm^2 and cultured for 4 days as described for the cell lines.

2.3. Preparation of cell extracts and [^{3}H]1,25-$(OH)_2D_3$ binding assay

For single point assays, conditions were employed which were previously shown to provide valid estimates of total receptor content in cytosolic extracts [6]. Cells were incubated and cultured as described above. Next, the cell pellet was extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodium molybdate and 0.1% Triton X-100. High-speed supernatants were obtained, and 250 μl aliquots were incubated overnight at 4°C, either for (1) single point assays with 0.25 nM [^{3}H]1,25-$(OH)_2D_3$ or (2) Scatchard analysis with several concentrations of [^{3}H]1,25-$(OH)_2D_3$ (ranging from 0.2 nM to 0.01 nM), in the absence or presence of a 200-fold molar excess of unlabeled hormone. Receptor-bound 1,25-$(OH)_2D_3$ was separated from unbound sterol by charcoal adsorption [21].

2.4. Osteocalcin assay

The incubation protocol to study the significance of TGFβ-induced increase in VDR level for the 1,25-$(OH)_2D_3$-stimulated osteocalcin production is shown in the

inset in Fig. 5A. Cells were preincubated with TGFβ for 4 h followed by the addition of 1,25-$(OH)_2D_3$ or vehicle without removal of TGFβ. Osteocalcin measurement in medium of MG-63 cells was performed by use of the INCSTAR (Stillwater, Minnesota, USA) ^{125}I RIA kit for osteocalcin. Before measurements, medium was freeze dried and reconstituted in assay buffer, so that medium was concentrated 6-fold. Cellular osteocalcin level of MG-63 cells was below detection limit. Osteocalcin measurements in cells and medium of ROS 17/2.8 cells were performed according to the method described by J. Verhaeghe et al. [22].

Sample preparation of cell extracts: cells were washed in PBS-triton X-100 (0.1%), removed in 0.5 ml 6 M guanidine-HCl in 0.1 M Tris-buffer (pH 8.0) and next assayed for osteocalcin.

2.5. DNA and protein measurements

Changes in DNA content were assessed by the fluorimetrical method of Johnson-Wint and Hollis [23]. Protein concentration was measured according to the method of Bradford [24].

2.6. Northern and dot blot analysis

RNA isolation was performed according to the method of Chomszinski [25]. Electrophoresis of total cytoplasmic RNA (30 μg) through a formaldehyde gel and Northern blotting (Gene Screen filters) were performed according to the method described by Davis et al. [26]. Dot blots were performed according to the method described by Sambrook et al. [27].

Hybridization of Northern blots: Northern blots were prehybridized for 2 h at 42°C in a buffer containing 50% formamide, 0.2% SDS, 1 × Denhardt's solution, 5 × NSSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate, pH 7.0), 20 mM NaH_2PO_4, 6% dextran sulphate, 1 μg/ml herring sperm DNA and then hybridized for 16–24 h at 42°C with ^{32}P-labeled cDNA probe. Filters were washed twice with 2 × SSC and 0.5% SDS for 5 min at room temperature, once with 2 × SSC and 0.5% SDS for 30 min at 50°C and once in 1 × SSC and 0.5% SDS for 30 min at room temperature, and exposed to X-ray films. The cDNA probes included 1.7 kb rat VDR fragment, 0.52 kb rat osteocalcin fragment, 1.3 kb rat osteopontin fragment, 1.2 kb hamster β-actin fragment, and 0.8 kb human GAPDH fragment. For rehybridization, filters were washed for 2 h at 65°C with 5 mM Tris-HCl (pH 8.0), 0.2 mM Na_2EDTA (pH 8.0), 0.05% sodiumpyrophosphate and 0.1 × Denhardt's solution.

2.6. Data analysis

To assess significance of interaction between two agents, tested data were analyzed with analysis of variance for 2-way design. Other statistical analyses were performed using Student's *t*-test.

3. Results

3.1. The effect of TGFβ on VDR level in several osteoblast-like cells

In UMR 106 (Fig. 1A), ROS 17/2.8 (Fig. 1B) and MG-63 (Fig. 1C) cells 1 ng/ml

TGFβ caused a time-dependent increase in 1,25-$(OH)_2D_3$ binding with a maximum after 6 h. After 24 h 1,25-$(OH)_2D_3$ binding decreased towards prestimulation level in all cell lines, although 1,25-$(OH)_2D_3$ binding was still significantly elevated in both ROS 17/2.8 and UMR 106 cells. As shown in Fig. 2, in all three cell lines tested,

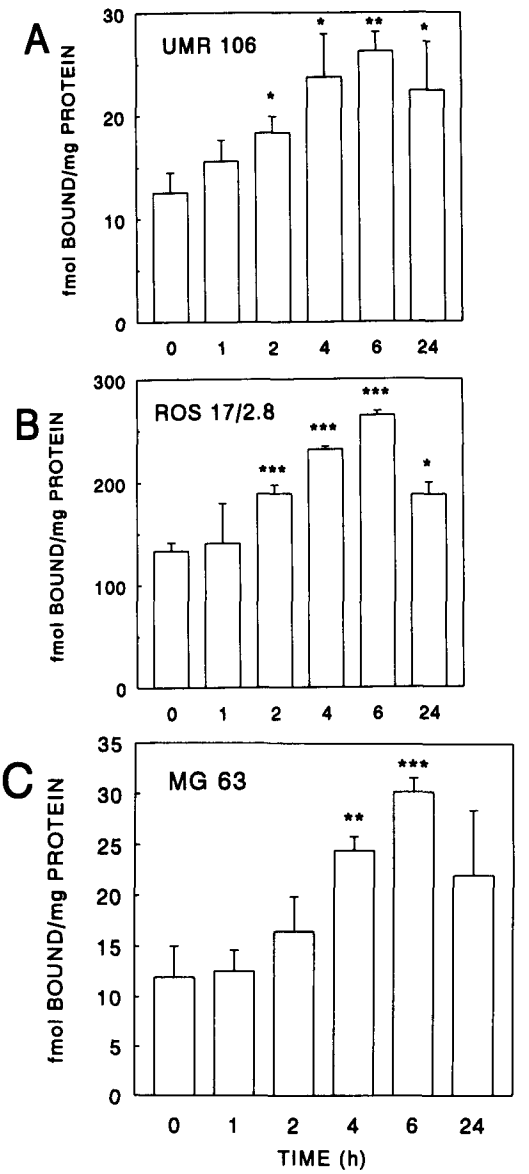

Fig. 1. Time-course of the effect of 1 ng/ml TGFβ on VDR level in (A) UMR 106, (B) ROS 17/2.8 and (C) MG-63 cells. Cells were cultured as described (Materials and methods) and incubated for the times indicated with 1 ng/ml TGFβ. Data are expressed as mean ± S.D. of single point assays in duplicate of two separate experiments. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ vs. control.

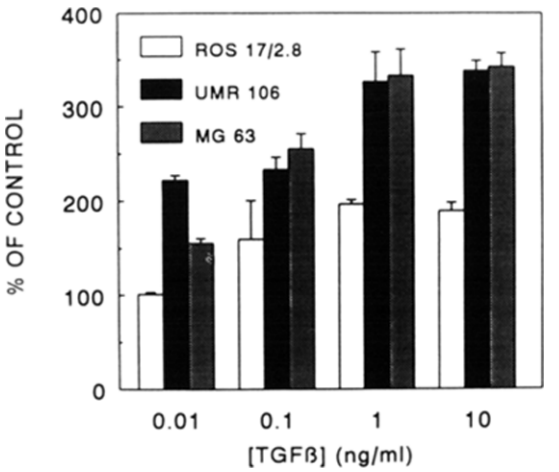

Fig. 2. Responses to different doses of TGFβ of VDR level in ROS 17/2.8 (open bars), UMR 106 (solid bars) and MG-63 (semi-solid bars) cells. Cells were cultured as described (Materials and methods) and incubated for 4 h with TGFβ. Data are expressed as percentage of control of single point assays (fmol bound/mg protein) in duplicate of two separate experiments. Increases in VDR level are significant ($P < 0.001$ vs. control) in all incubations, except for ROS 17/2.8 cells at 0.01 ng/ml TGFβ.

TGFβ caused a dose-dependent up-regulation of the VDR. A maximum effect was observed with 1 ng/ml TGFβ. In UMR 106 and MG-63 cells a significant increase was already observed after treatment with 0.01 ng/ml TGFβ, whereas in ROS 17/2.8 cells a first significant increase in VDR level was observed after incubation with 0.1 ng/ml TGFβ. The ED_{50} for the effect on VDR was 0.05–0.1 ng/ml in UMR 106 and MG-63 cells, and about 0.5 ng/ml in ROS 17/2.8.

In ROS 17/2.8 cells basal VDR level was higher than in MG-63 and UMR 106 cells. During the period we cultured the ROS 17/2.8 cells (about 20 passages) basal VDR decreased from 133.10 ± 8.22 fmol [^{3}H]1,25-$(OH)_2D_3$-bound/mg protein to 8.95 ± 0.49 fmol [^{3}H]1,25-$(OH)_2D_3$-bound/mg protein. However, independent of basal VDR level TGFβ (1 ng/ml) caused a twofold increase in VDR level (data not shown).

Besides these osteoblast-like cell lines also in isolated fetal rat osteoblasts TGFβ (1 ng/ml) increased 1,25-$(OH)_2D_3$ binding (from 16.51 ± 0.31 fmol [^{3}H]1,25-$((OH)_2D_3$-bound/mg protein to 29.29 ± 1.28 fmol [^{3}H]1,25-$(OH)_2D_3$-bound/mg protein). Both for the cell lines tested and the primary cultures the maximal increase in VDR level at 1 ng/ml TGFβ was 2–3-fold.

Scatchard analysis (Fig. 3) revealed that the increase in 1,25-$(OH)_2D_3$ binding in UMR 106 cells was not due to a change in the apparent dissociation constant of the VDR. An important role for new receptor synthesis in the TGFβ effect on VDR is further demonstrated by mRNA analysis. TGFβ increased VDR mRNA in ROS 17/2.8 (Figs. 4A and B) and UMR 106 cells (data not shown) in a dose- and time-dependent manner. The TGFβ-induced increase in VDR mRNA in ROS 17/2.8 cells was completely blocked during coincubation with the transcription inhibitor actinomycin D (1 μg/ml) (Fig. 4C).

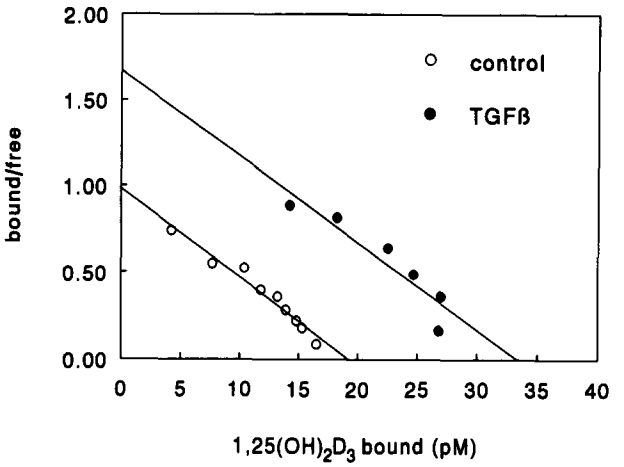

Fig. 3. Scatchard analysis of TGFβ effect on $1,25\text{-}(OH)_2D_3$ binding in UMR 106 cells. Cells were cultured as described (Materials and methods) and incubated for 24 h with or without 1 ng/ml TGFβ.

3.2. Effects of TGFβ pretreatment on 1,25-$(OH)_2D_3$ stimulation of osteocalcin production

To study the relation between receptor level and $1,25\text{-}(OH)_2D_3$ effects on osteocalcin production cells (ROS 17/2.8 and MG-63) were preincubated with TGFβ for 4 h (causing an increase in VDR level) and subsequently incubated with $1,25\text{-}(OH)_2D_3$ for 4 h and 24 h without removal of TGFβ. The incubation protocol is shown in diagram in the inset in Fig. 5A. The effects of 4 h preincubation with three concentrations of TGFβ (0.1, 1, 10 ng/ml) on subsequent stimulation (24 h) of osteocalcin production by various $1,25\text{-}(OH)_2D_3$ concentrations (10^{-11}–10^{-8} M) were studied. Cell proliferation was not affected by the continuous presence (28 h) of TGFβ in these experiments (data not shown). Basal osteocalcin production, measured in medium, was lower in MG-63 cells than in ROS 17/2.8 cells. In contrast to ROS 17/2.8 cells cellular osteocalcin content in MG-63 cells was below detection level. In both ROS 17/2.8 and MG-63 cells incubation with TGFβ (4 h preincubation with TGFβ followed by 24 h without addition of $1,25\text{-}(OH)_2D_3$) did not modulate basal osteocalcin production (Fig. 5A,B). $1,25\text{-}(OH)_2D_3$ caused a dose-dependent increase in osteocalcin production, which was not significantly affected by preincubation with 0.1 ng/ml TGFβ in both ROS 17/2.8 (data not shown) and MG-63 (Fig. 5A) cells. However, 1 ng/ml (ROS 17/2.8 and MG-63 cells) and 10 ng/ml TGFβ (ROS 17/2.8 cells), which doubled VDR level, inhibited the $1,25\text{-}(OH)_2D_3$ stimulation of osteocalcin production (Fig. 6A,B). Studies with ROS 17/2.8 cells showed that 10 ng/ml TGFβ was generally more potent than 1 ng/ml and that the effect of TGFβ is not the result of inhibition of osteocalcin secretion as it could be observed both in the medium and cellular extracts (data not shown). Time course studies with ROS 17/2.8 cells revealed that an inhibitory effect of TGFβ could be observed as

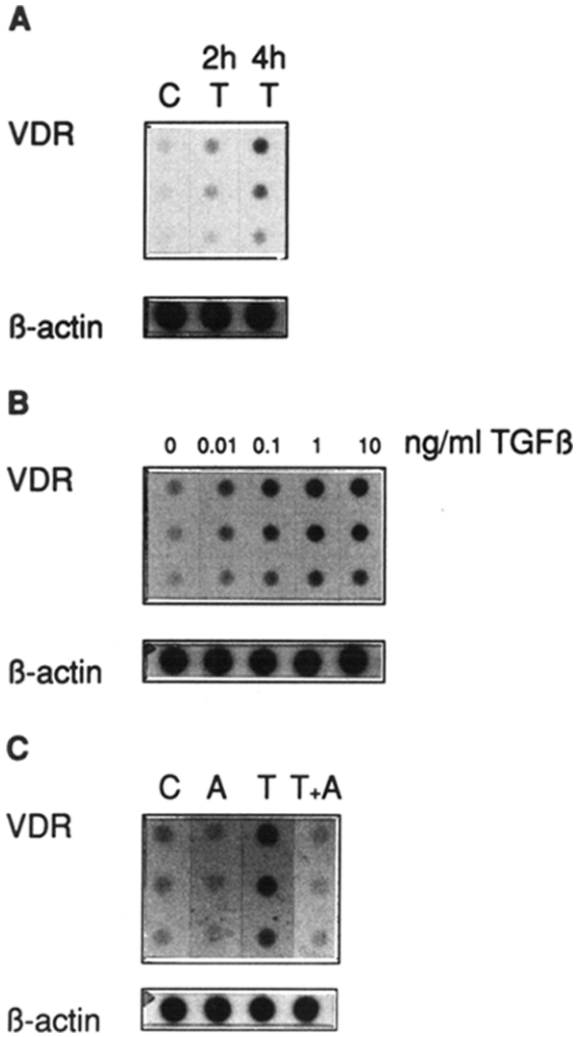

Fig. 4. Effect of TGFβ incubation on VDR mRNA in ROS 17/2.8 cells and the effect of coincubation with actinomycin D. Cells were cultured as described (Materials and methods) and incubated (A) for 2 and 4 h with 1 ng/ml TGFβ (T) or control (C) medium, or (B) for 4 h with various doses of TGFβ or (C) for 4 h with or without 1 ng/ml TGFβ (T) and 1 μg/ml actinomycin D (A). VDR mRNA data are shown in three dilutions of total RNA (15 μg, 5 μg and 1 μg). Actin mRNA reflects a representative of one of these dilutions.

soon as an effect of 1,25-$(OH)_2D_3$ on osteocalcin production was detected, i.e. in the cells after 8 h 1,25-$(OH)_2D_3$ incubation and in the medium after 16 h incubation (data not shown). The data on dissociation between TGFβ-induced up-regulation of VDR and 1,25-$(OH)_2D_3$ stimulation of osteocalcin production in ROS 17/2.8 and MG-63 cells are illustrated in Fig. 6A,B for stimulation of osteocalcin production by 10^{-8} M 1,25-$(OH)_2D_3$. Next, we studied the effect of TGFβ on the 1,25-$(OH)_2D_3$-induced increase in osteocalcin mRNA in ROS 17/2.8 cells. Basal osteo-

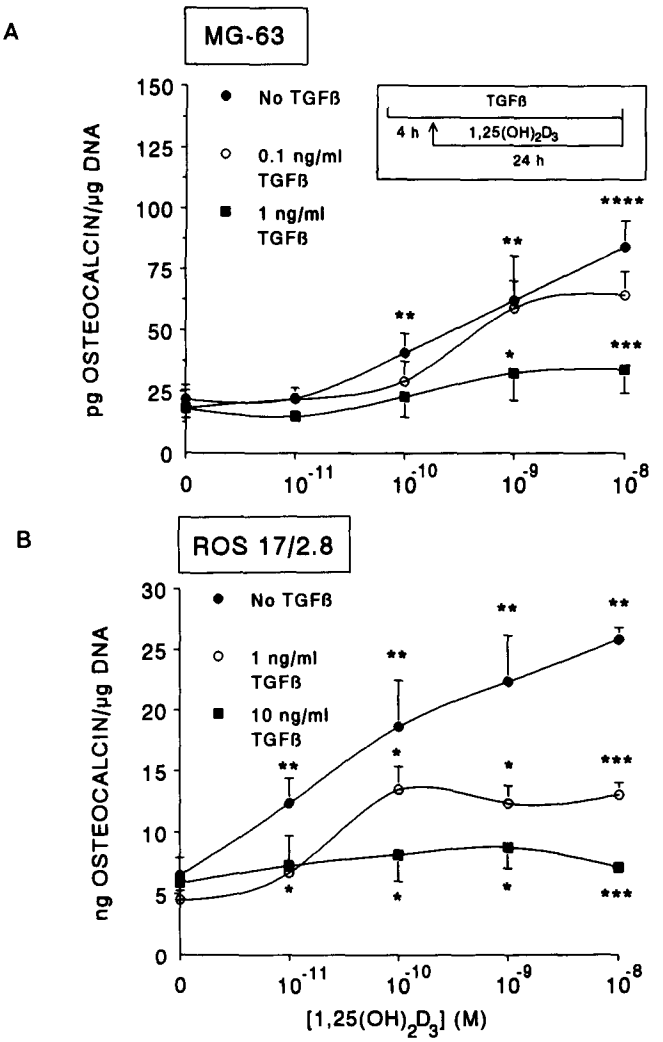

Fig. 5. The effect of preincubation with TGFβ on 1,25-$(OH)_2D_3$-induced increase in medium osteocalcin content of (A) MG-63 and (B) ROS 17/2.8 cells. Cells were cultured as described (Materials and methods) and incubated for 4 h with 0.1 or 1 ng/ml TGFβ and subsequently (without medium change) for 24 h (as illustrated in the inset in Fig. 5A) with several doses of 1,25-$(OH)_2D_3$. Data are expressed as mean ± S.D. of single point assays in duplicate of two separate experiments. $^*P < 0.05$ calculated as significance of interaction, $^{**}P < 0.005$ vs. control, $^{***}P < 0.005$ calculated as significance of interaction, $^{****}P < 0.001$ vs. control.

calcin mRNA was not affected after incubation with TGFβ for 8 h (4 h TGFβ preincubation followed by 4 h without 1,25-$(OH)_2D_3$) as well as after 28 h (4 h preincubation followed by 24 h without 1,25-$(OH)_2D_3$). Incubation for 24 h with 1,25-$(OH)_2D_3$ induced a significant increase in osteocalcin mRNA, whereas incubation for 4 h with 1,25-$(OH)_2D_3$ induced a small, non-significant increase in osteo-

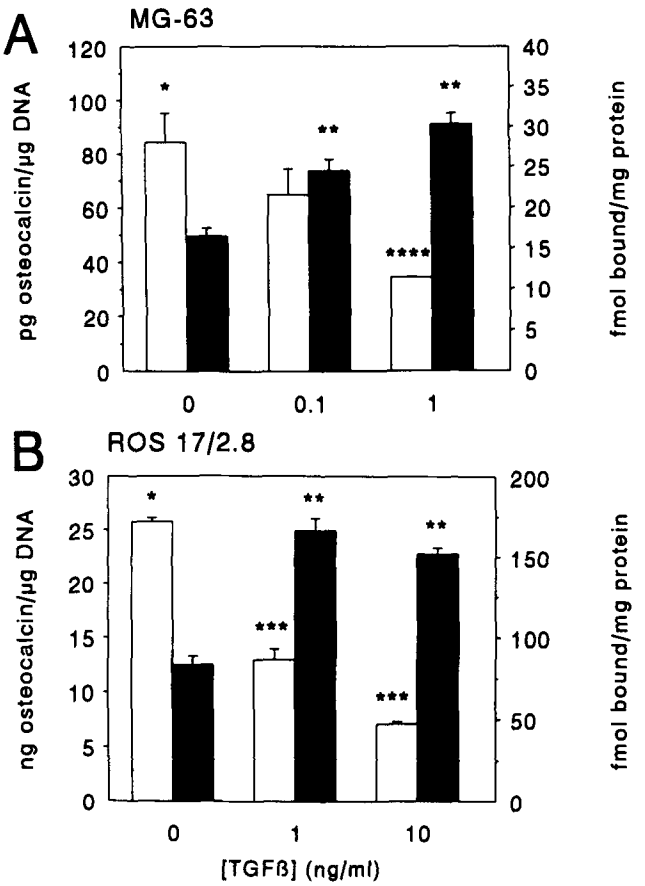

Fig. 6. Effect of TGFβ on VDR level in relation to its effect on 1,25-$(OH)_2D_3$-stimulated osteocalcin production in (A) MG-63 and (B) ROS 17/2.8 cells. Cells were cultured as described (Materials and methods) and incubated as shown in diagram in the inset of Fig. 5A. Data represent medium levels of osteocalcin (open bars) after 24-h incubation with 10 nM 1,25-$(OH)_2D_3$ with 4 h TGFβ preincubation and 1,25-$(OH)_2D_3$ binding data (solid bars) after 4 h of preincubation with TGFβ expressed as mean ± S.D. of single point assays in duplicate of two separate experiments. $^*P < 0.005$, $^{**}P < 0.001$ vs. control, $^{***}P < 0.001$ and $^{****}P < 0.005$ calculated as significance of interaction.

calcin mRNA (Fig. 7). Pretreatment for 4 h with 1 ng/ml TGFβ almost completely blocked the increase in osteocalcin mRNA by subsequent treatment with 1 nM 1,25-$(OH)_2D_3$ for 24 h. The 4 h 1,25-$(OH)_2D_3$ action was not significantly inhibited. However, there is certainly no potentiation of the 1,25-$(OH)_2D_3$ action as a result of the TGFβ-induced up-regulation of the vitamin D receptor. Generally, data obtained on mRNA level confirm the effects observed on protein level.

3.3. Effect of TGFβ pretreatment on 1,25-$(OH)_2D_3$ induction of osteopontin mRNA

Similar experiments as described for osteocalcin measurements were performed to study the effect of TGFβ pretreatment on 1,25-$(OH)_2D_3$-induction of osteopontin

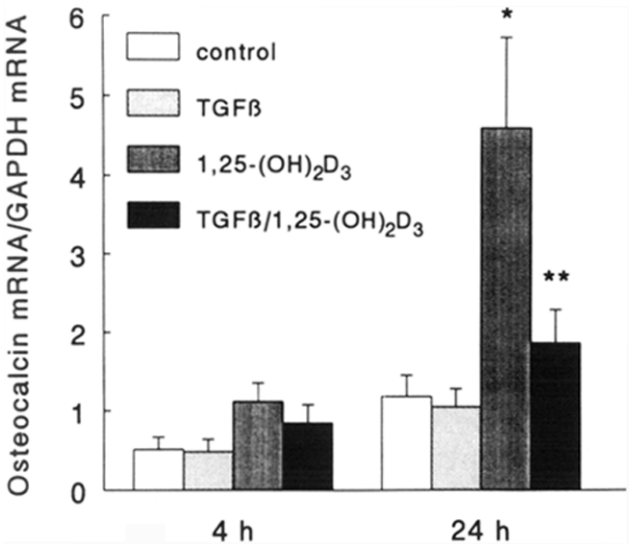

Fig. 7. Effect of preincubation with TGFβ on 4 and 24 h 1,25-$(OH)_2D_3$-increased osteocalcin mRNA in ROS 17/2.8 cells. Cells were cultured as described (Materials and methods) and incubated for 4 h with 1 ng/ml TGFβ and subsequently (without medium change) for 4 h and 24 h with 10^{-9} M 1,25-$(OH)_2D_3$. Northern blots were quantitated by densitometric scanning. Data are expressed as the ratio of the amount of osteocalcin mRNA to that of GAPDH mRNA. *$P < 0.05$ vs. control, **$P < 0.05$ calculated as significance of interaction.

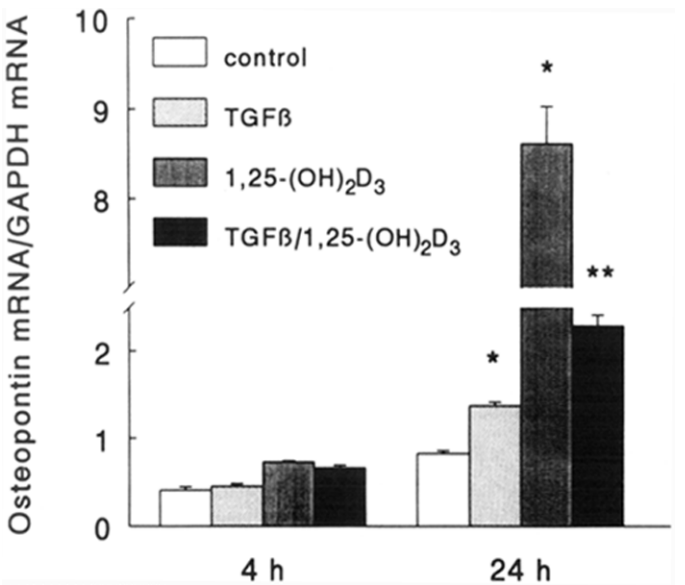

Fig. 8. The effect of preincubation with TGFβ on 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA in ROS 17/2.8 cells. Cells were cultured as described (Materials and methods) and incubated for 4 h with 1 ng/ml TGFβ and subsequently (without medium change) for 4 h and 24 h with 10^{-9} M 1,25-$(OH)_2D_3$. Northern blots were quantitated by densitometric scanning. Data are expressed as the ratio of the amount of osteopontin mRNA to that of GAPDH mRNA. *$P < 0.001$ vs. control, **$P < 0.001$ calculated as significance of interaction.

mRNA in ROS 17/2.8 cells. As shown in Fig. 8, 24-h 1 nM 1,25-$(OH)_2D_3$ induced an increase in osteopontin mRNA. Basal osteopontin mRNA was not affected after incubation with TGFβ for 28 h (4 h preincubation with TGFβ followed by 24 h without 1,25-$(OH)_2D_3$). A small, significant increase in osteopontin mRNA was observed. The 1,25-$(OH)_2D_3$-induced increase (24 h) was almost completely blocked by 4 h pretreatment with 1 ng/ml TGFβ. In short term incubations (4 h TGFβ followed by 4 h 1,25-$(OH)_2D_3$ incubation) both TGFβ and 1,25-$(OH)_2D_3$ did not affect osteopontin mRNA synthesis significantly (Fig. 8).

Together, as for the data on osteocalcin, the data obtained with respect to osteopontin after 24 h demonstrated a dissociation between TGFβ-induced up-regulation of VDR and the regulation of 1,25-$(OH)_2D_3$ biological activity (i.e. increased VDR level is not followed by a potentiation of 1,25-$(OH)_2D_3$ effects).

4. Discussion

VDR levels have been described to be regulated by several factors (5–12,28), including TGFβ [18]. In contrast to the data of Schneider et al. [18], who observed an increase after 72 h, we already observed a TGFβ effect on VDR level after 2 h with a maximum after 6 h. Our observations seem to represent a direct regulation of VDR level, whereas the data obtained by Schneider et al. may be the indirect result of changes in proliferation and differentiation of the cells. This is conceivable because TGFβ modulates the proliferation and differentiation of osteoblasts [29–33] and the VDR level has been reported to vary between developmental stages of cells [34,35]. However, in our experiments 28 h incubation with TGFβ did not affect proliferation. Our data show a comparable increase in VDR level by TGFβ in several osteoblast cell lines and in isolated fetal rat osteoblasts, which indicates that this TGFβ effect is not specific for transformed cells and is independent of species and state of differentiation. Data obtained with ROS 17/2.8 cells indicate that the effect of TGFβ is independent of basal VDR level. The increase in VDR level can be the result of (1) an increase in receptor affinity, (2) an increase in new receptor synthesis and/or (3) increase in half-life of the receptor or its mRNA. Scatchard analysis revealed that TGFβ increases the VDR number without a change in the apparent dissociation constant. The effect of actinomycin D on TGFβ-stimulated 1,25-$(OH)_2D_3$ binding indicates an effect on new synthesis of VDR rather than on VDR half-life and this is confirmed by mRNA analysis. Although we did not perform nuclear run-on experiments the fact that actinomycin D completely abolishes the TGFβ-induced increase in VDR mRNA level suggests that the TGFβ effect is mainly the result of stimulation of VDR gene transcription and not of an increased VDR mRNA half-life. However, additional post-transcriptional effects can not be excluded.

In both NIH 3T3 fibroblasts and ROS 17/2 cells TGFβ activates the α2(1) collagen promoter via a specific sequence in the promoter that is the binding site for nuclear factor I (NF1) [36]. In view of this, the current data suggest the presence of a TGFβ-responsive element or NF1 binding site in the VDR gene. However, as the sequence of the 5′-flanking region of the rat VDR gene is yet unknown it was not

possible to identify such sequences in the VDR gene. Also, within the known 115 nucleotides of the promoter region of the human VDR gene [37], no NF1 binding site could be detected. Kim et al. [38] demonstrated that TGFβ and phorbol ester act through the same site in the promoter of the TGFβ-gene. It is not likely that TGFβ and phorbol ester/protein kinase C act through the same site in the VDR gene as 4 h phorbol ester treatment results in a decrease [39], whereas TGFβ causes an increase in VDR mRNA.

For several 1,25-$(OH)_2D_3$ responses a relationship with VDR level has been described [8,12–14]. The present study shows that although TGFβ increases VDR level it potently inhibits 1,25-$(OH)_2D_3$-stimulated osteocalcin production (Fig. 6A,B). This dissociation is not unique for osteocalcin since the 1,25-$(OH)_2D_3$-induced increase in osteopontin mRNA was also inhibited by TGFβ (Fig. 8). Since we initially studied the effect of TGFβ preincubation on 24 h stimulation by 1,25-$(OH)_2D_3$, it was conceivable that during 24 h of incubation post-receptor mechanisms induced by TGFβ were more important than VDR up-regulation. These post-receptor mechanisms might be absent or less important in rapid responses to 1,25-$(OH)_2D_3$. However, time course studies revealed that an inhibitory effect of TGFβ could be observed as soon as an effect of 1,25-$(OH)_2D_3$ on osteocalcin production was detected. Although not yet significant, the inhibitory effect of TGFβ pretreatment on 1,25-$(OH)_2D_3$-induced increase in osteocalcin mRNA was already observed after 4 h incubation with 1,25-$(OH)_2D_3$. These data obtained with the TGFβ preincubation studies show an interaction between TGFβ and 1,25-$(OH)_2D_3$ with respect to osteocalcin and osteopontin mRNA synthesis at another level than VDR regulation. This is supported by the observations that 0.1 ng/ml TGFβ caused a significant increase in VDR level, whereas it did not affect 1,25-$(OH)_2D_3$-induced osteocalcin production.

Our results show interaction between TGFβ and 1,25-$(OH)_2D_3$ in the regulation of osteocalcin production. Osteocalcin is a chemo-attractant for human peripheral blood monocytes and macrophages [40,41] and data have been reported that osteocalcin is involved in recruitment and/or differentiation of bone resorbing cells [42,43]. Recently, it has been shown that TGFβ, at doses effective in the present study, inhibits 1,25-$(OH)_2D_3$-induced formation of osteoclast-like cells in human and mouse bone marrow cultures [44,45]. Combining these data from literature and the current data on the potent inhibitory effect of TGFβ on 1,25-$(OH)_2D_3$-stimulated osteocalcin production it is tempting to postulate that TGFβ inhibits 1,25-$(OH)_2D_3$-induced osteoclast formation via inhibition of 1,25-$(OH)_2D_3$-stimulated osteocalcin synthesis. Since osteopontin has been suggested to act as an anchor of osteoblasts to bone [46,47], our data on osteopontin support the idea of a negative interaction between TGFβ and 1,25-$(OH)_2D_3$ in the regulation of osteoclast action and are thereby in line with the osteocalcin data.

In conclusion the present study demonstrates a TGFβ-induced dissociation between VDR level and 1,25-$(OH)_2D_3$ action in osteoblast-like cells. At the moment the significance of VDR up-regulation by TGFβ remains unclear. Whether this is important for other 1,25-$(OH)_2D_3$ biological responses has to be established. More generally, the present study demonstrates the importance of the presence of locally

produced growth factors for the eventual response of target tissues/cells to hormone treatment.

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