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Transforming growth factor-β_1 downregulates dexamethasone-induced tetranectin gene expression during the in vitro mineralization of the human osteoblastic cell line SV-HFO

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Abstract In the present study, we examined the regulation of tetranectin gene expression using a human osteoblastic cell line, SV-HFO, that undergoes mineralization upon treatment with dexamethasone. We found that the expression of tetranectin and alkaline phosphatase mRNA was induced by dexamethasone treatment as evidenced by Northern blotting. When transforming growth factor-β_1 (TGF-β_1) was added together with dexamethasone to the SV-HFO cell cultures, the mineralization process was markedly suppressed and the expression of tetra nectin and alkaline phosphatase was downregulated in a dose-dependent manner. These results demonstrate that the expression of tetranectin in these osteoblastic cells is regulated by dexamethasone and TGF-β_1 and that tetranectin expression is tightly linked to the process of mineralization.

Key words: TGF-β_1; Tetranectin; Osteoblast; Mineralization

1. Introduction

Transforming growth factor β_1 (TGF-β_1) is a multifunctional compound that affects the proliferation, differentiation and gene expression pattern of several cell types, including osteoblasts [1–3]. Bone is an abundant source of TGF-β_1 [1,2] and in vivo studies have shown that TGF-β_1 is a local regulator of bone formation. In osteoblast cell culture systems, TGF-β_1 regulates the mineralization process and the expression of osteoblastic markers, including alkaline phosphatase, α_1 (I) procollagen, osteopontin, osteonectin and osteocalcin [4–22].

We have recently established a human osteoblastic cell line, SV-HFO, by immortalizing normal human fetal calvaria osteoblasts with simian virus 40 (SV-40) [23]. The cells had morphological and ultrastructural features characteristic of osteoblasts and produced low levels of osteoblastic markers like alkaline phosphatase and osteocalcin. The osteoblastic SV-HFO cells responded to osteotropic factors 1α,25-dihydroxyvitamin D_3, retinoic acid and TGF-β_1 [24]. The direct effect of TGF-β_1 on SV-HFO cells included a reduction of the amount of both alkaline phosphatase and osteocalcin [24]. More recently, we have shown that the cells undergo mineralization upon treatment with glucocorticoids [25]. In the present study, we used this human osteoblastic in vitro model system to begin analysing the regulation of tetranectin gene expression during mineralization. Tetranectin is a protein originally isolated from plasma [26] that was found to be expressed in bone at a time and space coincident with mineralization in vivo and in vitro [27]. We report that dexamethasone treatment induced the expression of tetranectin mRNA and that TGF-β_1 inhibited mineralization and the induction of tetranectin mRNA.

2. Materials and methods

2.1. Cell culture

The human osteoblastic SV-HFO cell line was established, cloned and maintained as previously described at 37°C in a humidified atmosphere of 95% air and 5% CO_2 [23]. The cells at passage 14 were seeded at a cell density of 1×10^4 cells/cm^2 on 35- or 100-mm culture dishes (Corning Glass Works, Corning, NY) in α-minimal essential medium (α-MEM; GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HR Bioscience, Lenexa, KS), 10 mM β-glycerophosphate (β-GP; Sigma, St Louis, MO), 100 U/ml penicillin and 100 μg/ml streptomycin (Immuno Biological Laboratories, Fujioka, Japan) and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (Hepes; Sigma). After 6 days of culture, the cells had reached confluence and dexamethasone (10^{-6} M; Orgadrone, Sankyo, Tokyo, Japan) was added to the cultures either alone or together with TGF-β_1 (human platelet-derived TGF-β_1; Sigma) at various concentrations (0.5 or 5 ng/ml). The cultures were maintained for another 7 or 21 days and then examined by von Kossa staining for minerals, calcium (Ca) and phosphate (P) measurements and used for the isolation of total RNA. The complete culture medium was renewed every 2–3 days.

2.2. Histochemical detection of minerals

The cells were rinsed with PBS, fixed in 20% formalin overnight and stained by the von Kossa technique for the detection of minerals [28].

2.3. Biochemical detection of minerals

The cells were rinsed with Hank's balanced salt solution (pH 7.4) and minerals deposited in the extracellular matrix were extracted with 0.8 ml of 5% perchloric acid at 4°C. The amount of Ca was measured spectrophotometrically by the *o*-cresolphtain Complexone method [29] and that of P was determined by the colorimetric method of Chen et al. [30].

2.4. RNA isolation and Northern blot analysis

Total RNA was isolated from cultures using the single-step guanidine thiocyanate-phenol-chloroform extraction method [31] with modifications as described [32]. For electrophoresis, 10 μg of total RNA was separated on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. The RNA was capillary blotted onto nylon membranes (Hybond-N;

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Amersham, Amersham, UK) in 20 × saline sodium citrate (SSC) and cross-linked with UV light. Human cDNA probes were as follows: α_1 (I) procollagen [33] kindly provided by Dr. Yasuko Koshihara (Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology); osteocalcin [34] was generously provided by Dr. Shintaro Nomura (Department of Pathology, Osaka University Medical School); alkaline phosphatase and TGF-β_1 from the American Type Culture Collection; and human tetranectin [35]. The probes were labeled with ^{32}P using multiprime DNA-labeling system kit (Amersham). The blots were prehybridized in a solution containing 50% formamide, 0.9 M NaCl, 0.1 M $NaPO_4$ (pH 7.4), 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml herring sperm DNA and 5 × Denhardt's solution at 42°C for 4 h and then hybridized overnight at 4°C in the same solution containing the ^{32}P-labeled probes. The membranes were exposed to Kodak Diagnostic film (Eastman Kodak, Rochester, NY) and an imaging plate and analysed using a Bio Imagining Analyser BAS 2000 (Fujix, Tokyo, Japan).

2.5. Statistical analysis

The data was statistically analysed using an unpaired, double-sided *t* test. All data were represented as mean values of four different dishes.

3. Results

The human osteoblastic cell line SV-HFO undergoes mineralization in vitro upon treatment with the glucocorticoid, dexamethasone (10^{-6} M) as demonstrated histochemically by von Kossa staining for minerals (Fig. 1A,B [25]). When confluent SV-HFO cells were grown for 21 days in the presence of dexamethasone and TGF-β_1 (5 ng/ml), a strong inhibition of mineralization was observed (Fig. 1C). This inhibitory effect of TGF-β_1 was quantitated by measuring the amount of deposited calcium (Ca) and phosphate (P) and was found to be dose-dependent. As demonstrated in Fig. 2, the addition of 0.5 ng/ml TGF-β_1 to the cultures reduced the amount of Ca and P by ~30% ($P < 0.001$) while 5 ng/ml of TGF-β_1 resulted in an almost complete inhibition ($P < 0.001$) of Ca and P deposition.

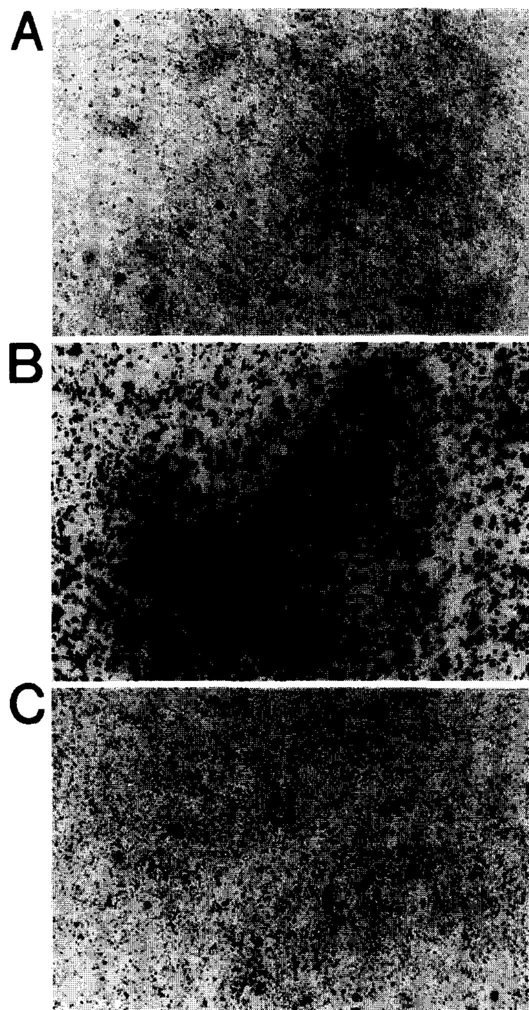

Fig. 1. Von Kossa staining for minerals of human osteoblastic SV-HFO cells cultured for 21 days without (A) or with dexamethasone (10^{-6} M) (B) or in the presence of both dexamethasone and TGF-β_1 (5 ng/ml) (C). Magnification × 27.

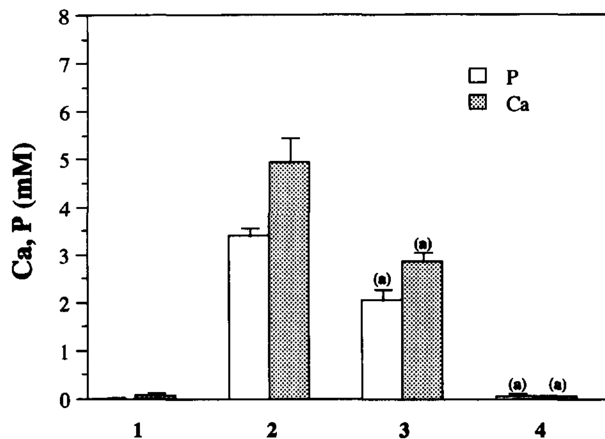

Fig. 2. Measurement of the amount in mM of calcium (Ca) and phosphate (P) in human osteoblastic SV-HFO cells cultured for 21 days without any treatment (1), in the presence of 10^{-6} M dexamethasone (2) or in the presence of 10^{-6} M dexamethasone combined with 0.5 (3) or 5 ng/ml (4) of TGF-β_1. The mean ± S.D. values of four different dishes with duplicate determinations are shown. (a) denotes that the values are significantly different from the values of cells cultured with dexamethasone alone; $P < 0.001$.

The influence of dexamethasone and TGF-β_1 on the expression of a series of bone marker proteins by the osteoblastic cells SV-HFO was examined by Northern blotting (Fig. 3A,B). The level of expression of alkaline phosphatase mRNA induced by dexamethasone treatment was downregulated by TGF-β_1 at concentrations of both 0.5 and 5 ng/ml (Fig. 3A) while the level of expression of α_1 (I) procollagen, osteocalcin and endogeneous TGF-β_1 in dexamethasone-treated cells appeared unaffected by TGF-β_1 (Fig. 3A). We then investigated the expression of tetranectin, a protein recently found to play a potential role in mineralization [27]. The expression of tetranectin mRNA was dramatically induced by the addition of dexamethasone (10^{-6} M) to the cell cultures (Fig. 3B, cf. lanes 1 with 2 and lanes 5 with 6). In cultures treated with both dexamethasone and 0.5 ng/ml TGF-β_1 for 7 days, tetranectin mRNA was present (Fig. 3B, lane 3) while, after 21 days of exposure to both compounds, no tetranectin mRNA could be detected (Fig. 3B, lane 6). When higher doses of TGF-β_1 (5 ng/ml) were applied, the induction of tetranectin mRNA was completely abolished at both time points (Fig. 3B, lanes 4 and 8), showing that the inhibitory effect of TGF-β_1 on the dexamethasone-induced expression of tetranectin mRNA was time- and dose-dependent.

4. Discussion

In this study, we have shown that the expression of tetra-

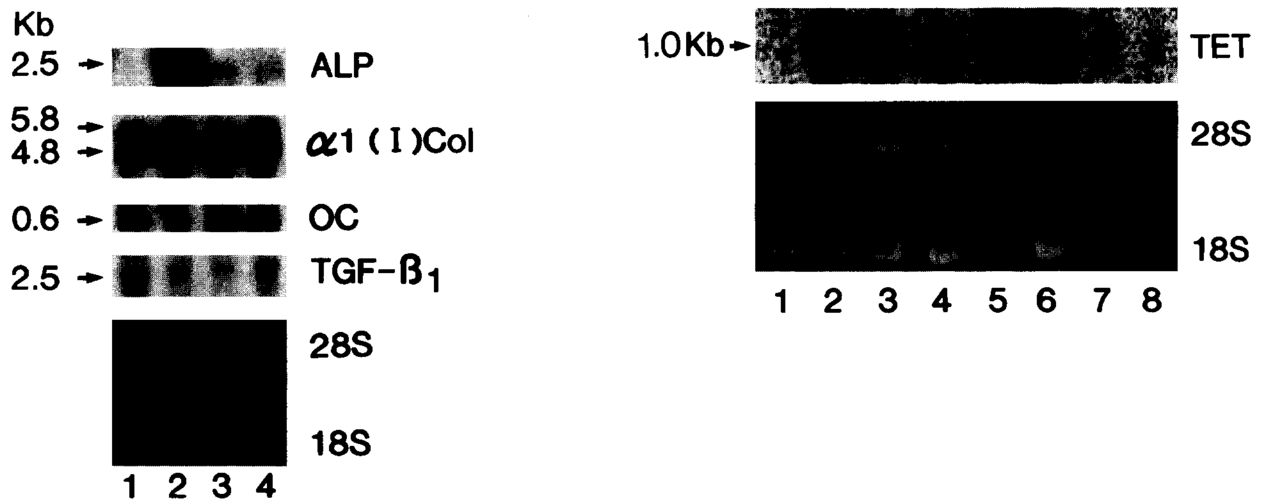

Fig. 3. (A, left) Northern blot analysis of mRNA for alkaline phosphatase (ALP), α_1 (I) procollagen (α_1(I)Col), osteocalcin (OC) and TGF-β_1 from human osteoblastic SV-HFO cells. Lane 1, no treatment; lane 2, 10^{-6} M dexamethasone; lane 3, 10^{-6} M dexamethasone and 0.5 ng/ml TGF-β_1; and lane 4, 10^{-6} M and 5 ng/ml TGF-β_1. (B, right) Northern blot analysis of tetranectin mRNA (TET) isolated from human osteoblastic SV-HFO cells cultured for 7 days (lanes 1–4) and 21 days (lanes 5–8) under the following conditions: lanes 1 and 5, no treatment; lanes 2 and 6, 10^{-6} M dexamethasone; lanes 3 and 7, 10^{-6} M dexamethasone and 0.5 ng/ml TGF-β_1; and lanes 4 and 8, 10^{-6} M dexamethasone and 5 ng/ml TGF-β_1. The sizes of the hybridizing mRNAs are indicated in kb. Lower panels, the ethidium-bromide stained RNA samples before transfer, ribosomal markers 18S and 28S are indicated.

nectin mRNA is upregulated during the dexamethasone-induced mineralization of the human osteoblastic cell line SV-HFO in vitro. This inductive effect of dexamethasone on tetranectin expression was inhibited by TGF-β_1 in a dose-dependent manner. The changes in tetranectin mRNA expression were paralleled by changes in alkaline phosphatase gene expression and the mineralization process. Our results provide new insight into the possible regulation of tetranectin mRNA expression during mineralization in osteogenesis and show that tetranectin expression is tightly correlated to the mineralization process.

Understanding the modulation of the osteoblastic phenotype is of interest in light of the its important role in, for example, the formation of new bone during fracture healing as well as in the maintenance of existing bone to prevent osteoporosis [2]. Furthermore, carcinoma cells have an intriguing effect on the bone metabolism, including the formation of osteolytic and osteosclerotic metastases [36]. It is becoming clear that the TGF-β supergene family plays an instrumental role for the osteoblastic cell lineage to display its full catalogue of phenotypic characteristics during osteogenesis. In various experimental systems for studying bone formation, contradictory results have been obtained on whether TGF-β_1 enhances or inhibits bone matrix formation, mineralization and the expression of bone marker proteins [4–22]. This has in part been explained by differences in the source and type of cells, stage of osteoblastic maturation, culture conditions and presence of other cytokines and their receptors. The cell system employed in the present study consists of an SV-40 immortalized human osteoblastic cell line (SV-HFO) [23] that undergo differentiation as evidenced by mineralization, upon treatment with dexamethasone [25]. Our results demonstrating that TGF-β_1 inhibits alkaline phosphatase expression and mineralization are consistent with several previous studies showing that TGF-β_1 appears to be an inhibitor of bone nodule formation and mineralization in osteoblast cultures [8–10,13,37]. A number of in vivo models have been established and it has been repeatedly shown that TGF-β injections stimulated bone formation [4–7]. However, endochondral ossification did not occur until after TGF-β injection had ceased [5]. Based on all these in vitro and in vivo studies, several authors suggested that, while TGF-β_1 initiates the proliferation, differentiation and extracellular matrix synthesis of bone cells, TGF-β_1 may be less involved in or actually inhibit the later phases of osteogenesis, i.e. mineralization [17,20,21]. The biological responses to TGF-β may be mediated by the retinoblastoma gene product [38], which binds to the SV-40 T antigen, and therefore the effect of T antigen expression by the SV-HFO cells on the TGF-β_1 signal transduction pathway will require further analysis.

The inhibition of mineralization of dexamethasone-treated SV-HFO cells by TGF-β_1 was associated with a strong inhibition of tetranectin gene expression as well as that of alkaline phosphatase. This demonstrates that tetranectin expression is tightly linked to mineralization, consistent with previous studies [27]. Although the exact biological function of tetranectin is unknown, it binds to the fourth kringle domain of plasminogen [25] and has a homology to C-type lectins which display calcium-dependent binding to carbohydrates [39,40]. The human and mouse cDNAs have been cloned [35,41,42] and the structure of the human tetranectin gene has been determined [43]. No information has yet been obtained on the regulation of the mouse or human tetranectin gene. The promoters of several of the bone-related genes contain an AP-1-binding site and for osteocalcin it has been demonstrated that this site may mediate the effect of TGF-β_1 by binding to the fos–jun complex [44]. Thus, it will be of interest to determine whether the tetranectin gene contains regulatory motifs similar to those found in other bone-related genes. Another aspect that merits further exploration is to test the effect of TGF-β_1 on tetranectin gene expression in other in vitro and in vivo bone model systems. In conclusion, our study provides the first evidence that the regulation of the tetranectin gene has important biological implications.

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