Osteotropic factor-stimulated synthesis of thrombospondin in rat dental pulp cells

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Abstract The amount of thrombospondin (TSP) mRNA in confluent clonal rat dental pulp cells was increased by transforming growth factor-β (TGF-β), 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and phorbol 12,13-didecanoate (PDD), with maximal levels 6, 36 and 2 h, respectively, after stimulation. These increases were accompanied by enhanced syntheses of TSP proteins which were found in the different forms in cell layer/matrix fraction (198 and 165 kDa TSP) and the culture medium (180 kDa TSP). These three factors also raised the mRNA level of osteopontin, which is thought to play an important role in mineralization in dentin and bone. The order of effectiveness of these factors was PDD > TGF-β > $1,25(OH)_2D_3$ for all the stimulations described above. These results suggest that the osteotropic factors enhance TSP synthesis at the pretranslational level and that TSP produced by dental pulp cells participates in formation of reparative dentin.

Key words: Thrombospondin; Dental pulp cell; Transforming growth factor-β; 1α,25-Dihydroxyvitamin D_3; Phorbol ester

1. Introduction

Thrombospondin (TSP) is a matricellular glycoprotein present in various cultured cells and animal tissues. This large trimeric protein is thought to be involved in a variety of biological activities and cellular events including platelet aggregation, adhesion, migration, differentiation, proliferation, angiogenesis, and phagocytosis [1–5], but its precise functions have not yet been defined at a molecular level.

Our preliminary experiments showed that TSP, which contains domains binding to Ca^{2+} and type I collagen [3], appeared in the calcification front of dentin. TSP has also been found in osteoid of subperiosteal bone [6], mineralized bone matrix [6], and enamel epithelium [7]. These findings suggest the participation of this protein in physiological calcification.

Dental pulp, an inside connective tissue of tooth, plays crucial roles in nourishment of odontoblasts and supplementary mineralization of dentin, reparative dentin being formed by dental pulp cells in response to external stimuli [8]. Active vitamin D_3 ($1,25(OH)_2D_3$) and phorbol ester stimulate synthesis of osteopontin (OPN), a phosphoprotein presumably related to mineralization, in rat dental pulp-derived clonal cells (RPC-C2A cell) [9,10].

This paper reports studies on the effects of osteotropic factors on TSP synthesis in RPC-C2A cells to investigate the possible roles of TSP and dental pulp cells in reparative calcification of dentin.

2. Materials and methods

2.1. Cell culture

RPC-C2A cells, a clonal cell line established from dental pulp of rat incisor [11], were kindly provided by Dr. S. Kasugai, Tokyo Medical and Dental University. These cells were grown in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) (HyClone) under a 5% CO_2 atmosphere and subcultured twice a week as reported [9]. The culture medium was changed every 2 days until the cells reached confluency. The confluent cells were used for all experiments in this study and were cultured in fresh EMEM containing 0.5% FBS for 12 h before addition of human transforming growth factor-β (TGF-β) (Boehringer), $1,25(OH)_2D_3$ (Biomol), or phorbol 12,13-didecanoate (PDD) (Sigma). RPC-C2A cells showed more than 90% viability even after cultured in the absence of FBS for up to 2 days. In this study, all experiments were repeated 2 or 3 times and typical results are shown in figures.

2.2. Northern blot analysis

A ^{32}P-labeled TSP cDNA fragment was prepared as follows. Plasmid pBT2E4 corresponding to TSP cDNA [12,13] was obtained in our laboratory from a cDNA library of calf odontoblasts with pBluescript II KS(+) as a vector. The *Hin*dIII–*Hin*cII fragment (0.92 kb) of plasmid-pBT2E4 encodes amino acid residues 686–990 of the total 1170 amino acids of TSP and contains the whole sequence complementary to highly conserved Ca^{2+}-binding repeats of TSP [3]. After purification by gel electrophoresis, the cDNA fragment was labeled with [α-^{32}P]dCTP (> 111 TBq/mmol; ICN) by random oligonucleotide-primed synthesis. Total RNA was isolated from guanidine thiocyanate-disrupted RPC-C2A cells by ultracentrifugation in cesium chloride solution [14]. A sample of 5 μg of RNA was fractionated in 1% agarose gel containing 6.5% formaldehyde [15]. After transfer to a Hybond-N^+ membrane (Amersham), and hybridization with cDNA probe ($1–5 \times 10^9$ cpm/μg), the membrane was washed with 1 × SSPE/0.1% SDS for 1 h and then with 0.1 × SSPE/0.1% SDS for 30 min [15]. The hybridized RNA was located by exposure to Fuji RX film at −80°C for 2 h with intensifying screens (DuPont). The same membranes were reprobed with cDNAs of mouse OPN and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [10]. Sizes of mRNAs were estimated with a 0.24–9.5 kb RNA ladder (Gibco BRL). The radioactivity of each band was also quantified with a Fujix Bioimaging Analyzer BAS-2000 II (Fuji).

2.3. Immunoradiometric assay of TSP synthesis

Labeling of protein with [^{35}S]methionine (185 kBq/ml, ICN) was carried out in methionine-free EMEM containing bovine serum albumin (0.25 mg/ml) for 6 h when a high level of TSP mRNA had been induced by an osteotropic factor. The confluent RPC-2CA cell/matrix layer and the culture medium were assayed separately as follows. The

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Abbreviations: TSP, thrombospondin; OPN, osteopontin; TGF-β, transforming growth factor-β; $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PDD, 4β-phorbol 12,13-didecanoate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EMEM, Eagle's minimum essential medium; PKC, protein kinase C; SDS-PAGE, SDS polyacrylamide gel electrophoresis; VDRE, vitamin D response element

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cell/matrix layer in culture dishes (6 cm diameter) was lysed in RIPA buffer [16] containing aprotinin (0.23 U/ml), (*p*-amidinophenyl) methanesulfonyl fluoride (0.1 mM), leupeptin (10 μg/ml), and pepstatin (10 μg/ml). Protein in the lysate was determined with a protein assay kit (Bio-Rad). The culture medium (4 ml) was concentrated to 0.2 ml in a Centricon 30 (Amicon) in the presence of the four protease inhibitors described above. A sample of cell lysate containing 50 μg protein or 0.15 ml of medium concentrate was mixed with 2.5 μg of monoclonal anti-human TSP antibody 5G11 (Immunotech) [17] and incubated at 4°C for 12 h. The immune complex was precipitated with 50 μl of protein G (10% Group C *Streptococcus* sp. cell suspension, Sigma), boiled in Laemmli's SDS sample buffer [18] and analyzed in 7.5% polyacrylamide discontinuous SDS gel [18]. The gel was dried and radioactivity was detected as described above. Molecular-weight markers (HMW; Kit E for SDS-PAGE, Pharmacia) were used as standards.

3. Results

Under the experimental conditions employed, confluent RPC-C2A cells showed very low but constant mRNA levels of both TSP and OPN during the entire experimental period (48 h). As shown in Fig. 1, the mRNA level of TSP began to increase within 2 h after addition of TGF-β, reached a maximum at 6 h and then decreased, even in the presence of TGF-β. When measured with a Fujix Bioimaging Analyzer, the maximal level was 5 times the control. This increase was dose-dependent with 1–10 ng/ml of TGF-β (data not shown). RPC-C2A cells contain two forms of TSP transcripts of 3.61 kb and 5.78 kb, the latter being predominant (65% of the total TSP mRNA), and these two transcripts increased similarly on treatment with TGF-β. TGF-β also raised the mRNA level of OPN from 4 to 24 h after stimulation. Its maximum level at 12 h was 3 times the control level (Fig. 1) but returned to the control level at 36 h (data not shown). These time-course experiments indicated that the response of TSP to TGF-β was more pronounced and earlier than that of OPN. The mRNA level of GAPDH was not significantly changed at any time after treatment with the three stimulants used in this study (Figs. 1–3).

$1,25(OH)_2D_3$ also induced increases in the mRNAs of both TSP and OPN with similar time courses (Fig. 2A). Their maximal levels at 36 h were respectively 2.6 and 11.0 times their control levels. There are reports that the *OPN* gene has a vitamin D response element (VDRE) in its upstream sequence [19,20] but that the *TSP* gene has not [21] and that $1,25(OH)_2D_3$ exerts its effects through genomic or non-genomic actions. In the latter case, $1,25(OH)_2D_3$ activates phospholipase C which produces diacylglycerol, an activator of protein kinase C (PKC), through hydrolysis of phosphoinositides [22]. Therefore, we examined the effects of calphostin C, a specific inhibitor of PKC [23] and of PDD, a PKC activator, on the $1,25(OH)_2D_3$-dependent expressions of TSP and OPN. As shown in Fig. 2B, calphostin C at concentrations of 0.02 and 0.1 μM caused 47 and 91% inhibitions of $1,25(OH)_2D_3$-enhanced TSP expressions, respectively. Consistent with this result, 10^{-8} or 10^{-7} M PDD markedly increased the level of TSP mRNA from 1 to 10 h after stimulation, the maximum level being 6 times the control level at 2–4 h (Fig. 3). PDD also increased OPN mRNA with the same time course and dose-dependency as TSP mRNA (Fig. 3). On the contrary, calphostin C did not inhibit the $1,25(OH)_2D_3$-enhanced OPN expression or the TGF-β-dependent expressions of TSP and OPN (Fig. 2B), indicating specific inhibition of $1,25(OH)_2D_3$-induced TSP expression by calphostin C.

Immunoradiometric experiments revealed the presence of TSPs of two sizes (198 and 165 kDa) in the cell/matrix fraction (Fig. 4A) and that of another size (180 kDa) in the culture medium (Fig. 4B) of RPC-C2A cells. Under our experimental conditions, the rates of syntheses of these three TSP proteins were increased almost equally by the stimulants and the increases were roughly proportional to those of the TSP mRNA levels. The increases were 4.5, 3.5 and 6.0 times the control levels with TGF-β, $1,25(OH)_2D_3$ and PDD, respectively.

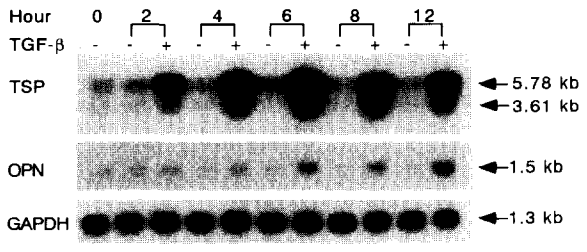

Fig. 1. TGF-β-induced elevation of mRNA levels of TSP and OPN in rat dental pulp cells. TGF-β (10 ng/ml) was added at 0 h to the culture medium of confluent RPC-C2A cells and RNA was extracted from the cells at the indicated times. Other procedures are described in Section 2.

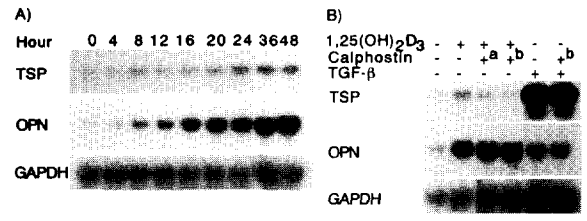

Fig. 2. Effects of $1,25(OH)_2D_3$ and calphostin C on mRNA levels of TSP and OPN. (A) Time course experiment: $1,25(OH)_2D_3$ (10^{-8} M) was added at 0 h and RNA was extracted at the indicated times. (B) Effects of calphostin C: calphostin C (0.02 μM[a] or 0.1 μM[b]) was added 30 min before addition of $1,25(OH)_2D_3$ (10^{-8} M) or TGF-β (10 ng/ml) and RNA was extracted 16 and 6 h after addition of the former and the latter, respectively.

4. Discussion

TGF-β and $1,25(OH)_2D_3$ are endogenous osteotropic factors which play regulatory roles in development of tooth and bone. With respect to tooth, TGF-β has been found in the dentin matrix and developing molar teeth [24] and has been suggested to be released and affect functions and differentia-

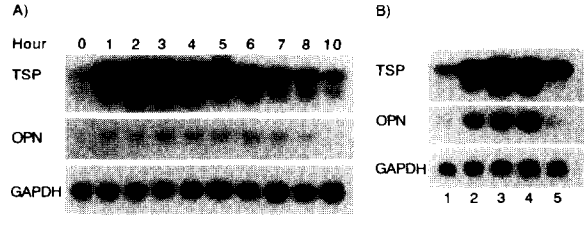

Fig. 3. Effects of PDD on mRNA levels of TSP and OPN. (A) Time course experiment: PDD (10^{-8} M) was added at 0 h and RNA was extracted at the indicated times. (B) Dose dependency: RNA was extracted 2 h after addition of the following dose of PDD: 1, 0; 2, 10^{-9} M; 3, 10^{-8} M; 4, 10^{-7} M; 5, 10^{-6} M.

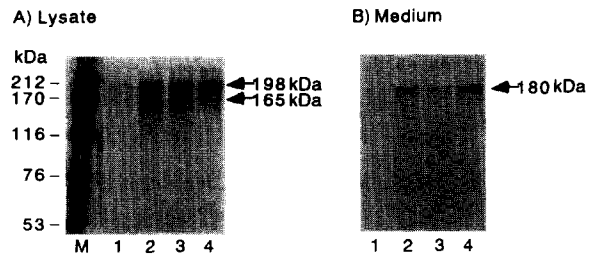

Fig. 4. Effects of osteotropic factors on the synthesis of TSP protein. Confluent RPC-C2A cells were cultured with [^{35}S]methionine for 6 h when the level of TSP mRNA was high in the absence (control) or presence of an osteotropic factor which was added at 0 h. TSP in the cell/matrix lysate (A) and culture medium (B) were separately assayed as described in Section 2. The labeling times were as follows; (1) Control, 0–6 h. This control value was almost constant during the entire experimental period (48 h). (2) TGF-β (10 ng/ml), 2–8 h. (3) $1,25(OH)_2D_3$, 36–42 h. (4) PDD (10^{-8}M), 0–6 h. M, molecular-weight markers.

tion of pulp cells and odontoblasts [25] during repair after caries and dental treatments [26]. Experimental [27] and clinical data [28] have also shown the participation of $1,25(OH)_2D_3$ in dentin mineralization. The RPC-C2A cells used in this study are clonal fibroblast-like cells derived from the deep part of rat incisor pulp. This cell line has high activity of alkaline phosphatase, a marker enzyme of odontoblasts [29], but the role of these cells in dentin mineralization is still unknown. In this study, we showed that both TGF-β and $1,25(OH)_2D_3$ increased the mRNA levels of TSP and OPN and that the increase in TSP mRNA was followed by enhanced synthesis of TSP protein (Figs. 1, 2 and 4). Nagata et al. [10] reported that the elevated level of OPN mRNA induced by $1,25(OH)_2D_3$ was associated with increased synthesis of OPN protein in RPC-C2A cells. Although $1,25(OH)_2D_3$ increased the expressions of TSP and OPN with similar time courses (Fig. 2), the effects of calphostin C suggested that the expressions of TSP and OPN were due to PKC-mediated and VDRE-mediated actions of $1,25(OH)_2D_3$, respectively, consistent with the fact that VDRE is located upstream of the *OPN* gene [19,20] but not the *TSP* gene [21]. The PDD-dependent expressions of TSP and OPN (Fig. 3) were also consistent with the presence of AP-1 and AP-2 sites in both genes [20,21]. The stimulatory effects of PDD appeared earliest and were strongest, the order of effectiveness being PDD > TGF-β > $1,25(OH)_2D_3$ for the mRNA levels of TSP and OPN and the rate of TSP synthesis. These results suggest that the three factors stimulate TSP synthesis at the pretranslational level. The $1,25(OH)_2D_3$-induced PKC-mediated expression of TSP mRNA showed a similar time course to its genomic OPN expression (Fig. 2) and was more than 30 h later than that by PDD. The reason for this delay requires study.

There are reports of isolation of TSP from unstimulated and stimulated osteoblasts [30], monocyte-like cells [31] and endothelial cells [32] by SDS-PAGE, but none of them clearly showed different sizes of cellular and secreted TSP. We found for the first time TSP proteins of two sizes (198 and 165 kDa) in cell lysates and a further one (180 kDa) in the culture medium (Fig. 4) in addition to TSP mRNAs of two sizes (Figs. 1–3). The significances of these multiforms of TSP mRNA and protein remain to be studied. Possibly the three forms of TSP have different biological functions because TSP is a typical multifunctional protein [1–5].

Kasugai et al. [33], Roach [34] and Yao et al. [35] reported that OPN and bone sialoprotein are important matrix proteins in the mineralization process. TSP, OPN and bone sialoprotein have the following characteristics in common. These glycoproteins all contain sequences of RGD and polyaspartate or polyglutamate [3,36,37], have high binding capacities to vitronectin receptor, collagen and hydroxyapatite or Ca^{2+} ion [5,33–36] and are localized in front of regions of mineralization and calcified matrix [6,34]. In this connection, it is noteworthy that we found TSP in the predentin area of calf deciduous anterior teeth (unpublished data). In addition to these facts, the similar responsiveness of TSP and OPN to osteogenetic factors shown in this study suggests that TSP produced by dental pulp RPC-2CA cells participates in supplementary mineralization in dentin restoration.

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