RESEARCH REPORTS
Biological

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DPP and DSP are Necessary for Maintaining TGF-β1 Activity in Dentin

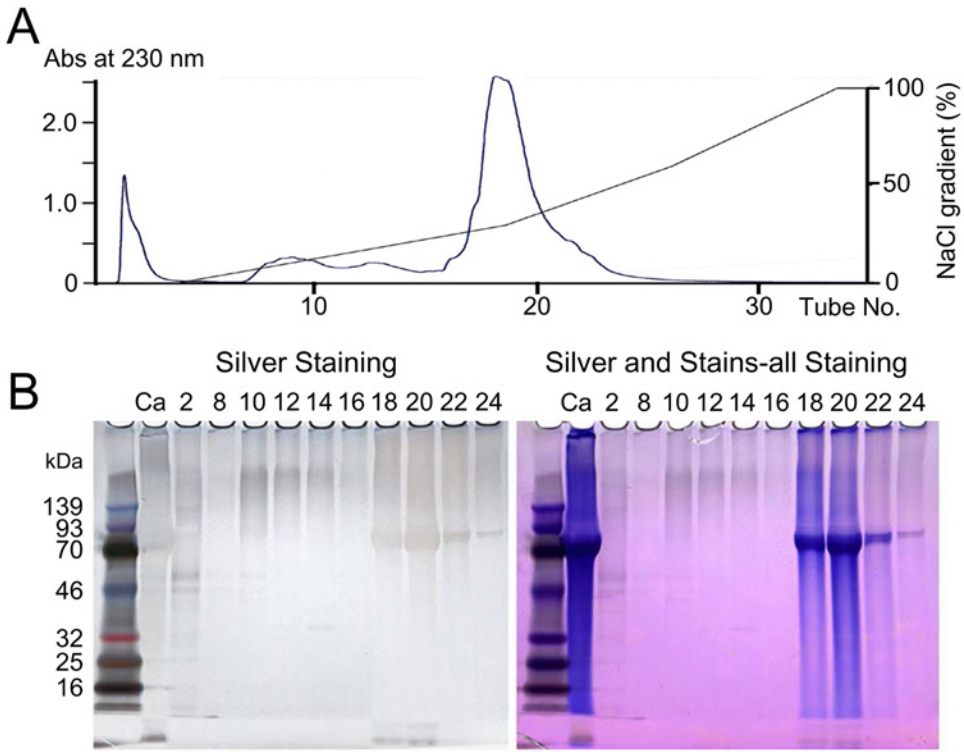
J Dent Res DOI: 10.1177/0022034514534690

APPENDIX

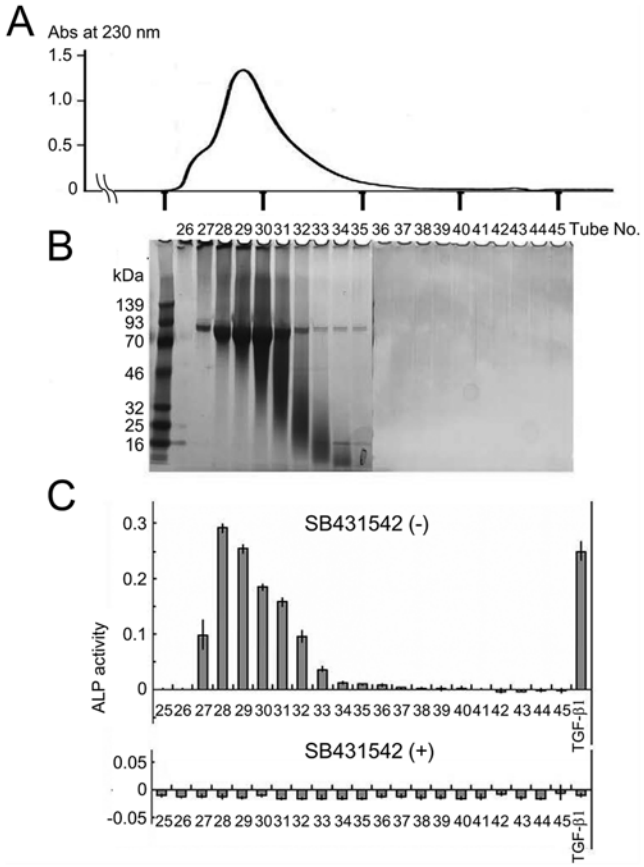
Human periodontal ligament (HPDL) cells possess the multipotency to undergo differentiation into fibroblasts, osteoblasts, and cementoblasts. HPDL cells have higher levels of alkaline phosphatase, which is a marker enzyme for differentiated osteoblasts (Somerman *et al.*, 1988; Kawase *et al.*, 1990). Our previous study demonstrated that TGF-β in both Emdogain gel and porcine immature enamel extracts enhanced alkaline phosphatase (ALP)-stimulating activity in HPDL cells and induced them to express an osteoblast-like phenotype, such as up-regulated osteocalcin and bone sialoprotein (BSP) mRNA expression, and the formation of mineralized nodules in the differentiation medium (Nagano *et al.*, 2004, 2006; Suzuki *et al.*, 2005). In this study, we used the same HPDL cell culture system, designated "ALP-HPDL system", to evaluate the osteoinductive ability by TGF-β in the dentin matrix of both porcine incisors and molars.

The TGF-β is found in ethylenediaminetetraacetic acid (EDTA)-soluble fraction in rabbit and human incisor dentin (Finkelman *et al.*, 1990; Bègue-Kirn *et al.*, 1992; Cassidy *et al.*, 1997; Baker *et al*., 2009). We first decided to use porcine incisor dentin for our preliminary study. A quantity of about 90 mg of tryptone glucose extract (TGE) was obtained from 15 g of the incisor dentin of porcine developing teeth by extraction with 4 M guanidine/0.5 M EDTA buffer. The dentin phosphoprotein (DPP) fraction obtained by means of Ca precipitation from TGE extracts yielded about 60 mg. When the DPP fraction was purified by diethylaminoethanol (DEAE) ion exchange high-performance liquid chromatography (HPLC) and monitored by SDS-PAGE (Appendix Fig. 1), DPP eluted from 0.3 to 0.6 M NaCl concentration, and was visualized as a purple band by silver and Stains-All double-staining. We further purified the DPP fractionated by size exclusion HPLC using double tandem columns of TSK gel G3000PW equilibrated in 4 M guanidine (Appendix Fig. 2A). The fractions contained no distinct protein bands by silver staining (data not shown), but stained with Stains-All (Appendix Fig. 2B). After the fractions were de-salted, their ALP-stimulating activity was examined in the HPDL cell culture system. The ALP-stimulating activity was detected along with the elution of DPP, but it peaked in a fraction that eluted a little earlier than DPP (Appendix Fig. 2C). We demonstrated that TGF-β was extracted with guanidine-EDTA buffer and co-purified with DPP by both ion exchange and size exclusion HPLCs. It was confirmed by the determination of ALP-stimulating activity in HPDL cells that even the DPP purified by the size exclusion HPLC in 4 M guanidine buffer (under denaturing conditions) has osteoinductive activity that was completely inhibited by the TGF-β specific inhibitor.

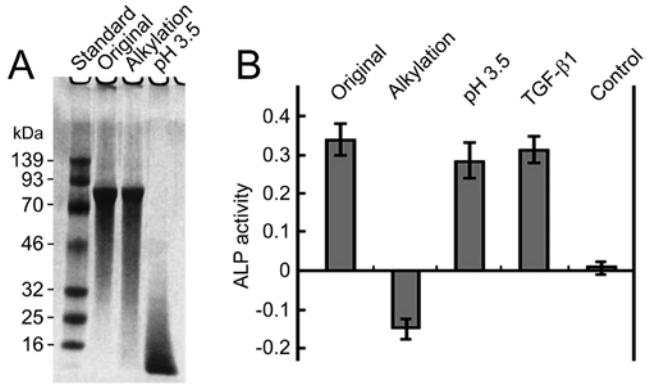
The purified DPP obtained by size exclusion HPLC was incubated under 2 different conditions (Appendix Fig. 3A). When the DPP was reduced with β-mercaptoethanol and alkylated with iodoacetic acid, it appeared to be intact on SDS-PAGE, but showed no ALP-stimulating activity (Appendix Fig. 3B). When the DPP was incubated at pH 3.5 for 8 hr at 60°C, it was degraded into lower-molecular-weight species that migrated at the dye front on SDS-PAGE (Appendix Fig. 3A), but the ALP-stimulating activity of this fraction was nearly undiminished by this incubation (Appendix Fig. 3B). When the recombinant TGF-β was added to the ALP-HPDL system, its ALP-stimulating activity was the same as when DPP was originally added (Appendix Fig. 3B). TGF-βs are synthesized as either a small latent complex consisting of a mature dimeric TGF-β, non-covalently bound to a latency-associated protein (LAP), or as a large latent complex consisting of the dimeric growth factor and LAP covalently cross-linked through disulfide bonds to latent TGF-β binding protein (LTBP). The proteolytic cleavage required for activation to the mature TGF-β is a homodimer of its carboxy terminal domain connected by the disulfide bond (Hyytiäinen *et al.,* 2004). When the DPP was treated with mercaptoethanol, DPP was not affected by this reduction, because there are no cysteines in the DPP amino acid sequence. But this reaction produced complete reduction of ALP-stimulating activity of DPP. Our previous study showed that the DPP is unstable at low pH and high temperatures because it is an intrinsically disordered protein (Yamakoshi *et al.*, 2008). When DPP was treated in pH 3.5 at 60°C for 8 hr, DPP completely degraded, but ALP-stimulating activity was retained in HPDL cells. This finding suggested that the ALP-stimulating activity was not due directly to the DPP, but was due to the residence of TGF-β even in the purification by size



Appendix Figure 1. Isolation of dentin phosphoprotein (DPP) in porcine incisor dentin. **(A)** DEAE IE-HPLC showing absorbance at 230 nm for TGE extracts after Ca precipitation from dentin of porcine developing incisors (60 mg). **(B)** SDS-PAGE (15% gel) stained with Silver only and Silver and Stains-All double-stain showing original TGE extracts after Ca precipitation (Ca) and each tube on a DEAE chromatogram.



Appendix Figure 2. Isolation of TGF-β-bound DPP in porcine incisor dentin. **(A)** TSK-gel G3000PW size exclusion HPLC showing absorbance 230 nm for DPP sample (6 mg) fractionated by DEAE IE-HPLC. **(B)** SDS-PAGE (15% gel) stained with Stains-All showing each tube on size exclusion chromatogram. **(C)** ALP-inducing activity of HPDL cells exposed by each tube and TGF-β1 (0.3 ng/mL) without (-) or with (+) the TGF-β1 receptor inhibitor, SB431542, at a final concentration of 1 mM. Data are means ± SE of 3 culture wells.



Appendix Figure 3. Effect of DPP protein and TGF-β activity bound on DPP under alkylation or low pH and high temperature. **(A)** SDS-PAGE (15% gel) stained with Stains-All and (**B**) ALP-inducing activity of HPDL cells show: molecular-weight marker (Standard), purified DPP (Original), DPP after alkylation (Alkylation), DPP after incubation under pH 3.5 at 60°C (pH 3.5), TGF-β1 (0.3 ng/mL) (TGF-β1), and acetic acid solution (5.2 mM) only (Control). Data are means ± SE of 3 culture wells.

Appendix Figure 4. LC-MS/MS analysis of an approximately 13-kDa protein band in osteoinductive fractions in ANQ3 and ANQ4. (**A**) SDS-PAGE (4% to 12% gradient gel) stained with Silver showing osteoinductive fractions in ANQ3 and ANQ4. The protein bands having the molecular weight of approximately 13 kDa (arrows) were excised from the gel and were analyzed by LC-MS/MS. (**B**) Amino acid sequence of porcine TGF-β1. The bold and underlined sequence indicates the region identified by LC-MS/MS analysis.

exclusion HPLC in the denatured condition. In other words, the ALP-stimulating activity in DPP fraction obtained from the size exclusion HPLC in the denatured condition is due to the TGF-β bound to DPP in porcine incisor dentin.

In this study, we used porcine molars to obtain DPP containing TGF-β activity in quantity from developing pig teeth and confirmed that DPP in porcine molars behaves the same as DPP isolated from the incisor.

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