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Article

TGF-β and Physiological Root Resorption of Deciduous Teeth

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Appendix

The TGF-β activity has been found in the pulp and periodontal ligaments [1]. Because the denaturing reagent was used for that study, we modified the extraction procedure for TGF-β by using a combination of non-ionic detergent; such as Triton X and NP-40, and subsequent ionic detergent SDS in the present study. The amount of total protein in each sample was quantitatively determined with Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The collected tissue weights from each group, the total micrograms of protein in each sample, and the micrograms of protein extracted per milligrams of tissue are provided in Table A1. The amount of protein was normalized for each sample on total milligrams of tissue basis and was applied per lane for SDS-PAGE (Figure 1C).

The quantitative real-time PCR (qPCR) was performed using the SYBR Green technique on a LightCycler Nano (Roche Diagnostics) against purified total RNA prepared from the root-surrounding tissues at R1-R3 and N1-N3 regions. The selected primers, running conditions and size of amplified product are provided in Table A2. Each ratio was normalized the relative quantification data of TGF-β1, TGF-β3, RANKL, RANK, TRAP, CALCR, NFATc1 and OPG in comparison to a reference gene (GAPDH) was generated on the basis of a mathematical model for relative quantification in qPCR system.

Odontoclasts possess similar characteristic to osteoclasts, such as the ultrastructure, and enzymatic and metabolic properties to those of the osteoclasts [2-5]. RAW264 cells have been widely used for osteoclast differentiation experiment. Therefore, we used RAW264 cells in the present study and considered the differentiation toward osteoclasts as that toward odontoclasts. We tested if RANKL stimulated RAW264 cells have the ability to induce the osteoclast by three TGF-β isoforms; TGF-β1, TGF-β2 and TGF-β3 (Figure 4A and 4B). To support this test, we also examined the induced TRAP (iTRAP) activity for RANKL stimulated cells with OPG. The iTRAP activity in cells was observed to decrease in a concentration-dependent manner of OPG (Figure A1). The findings of TRAP staining for osteoclasts were compared with those of controls without OPG. The level of TRAP staining of osteoclasts containing OPG decreased in a concentration-dependent manner of OPG (Figure A1). Thus, we confirmed that three recombinant human TGF-βs isoforms evenly induced the RANKL-mediated osteoclast differentiation and the addition of OPG suppressed it. Then, we used this RAW264 cells for proving that *in vivo* TGF-βs in Triton X fraction induce RANKL-mediated

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osteoclast differentiation (Figure 3C).

Table A1. Collected tissue weight, raw data from 660 nm protein assay, the total micrograms of protein in each sample, and the micrograms of protein extracted per milligrams of tissue.

Triton X	Tissue (mg)	660 assay (µg/ml)	Total (µg/µl)	µg/mg tissue
N1	106.3	5450	2779.5/510	26.1
N2	99.45	4700	3543.8/754	35.6
N3	109.2	6500	4680/720	42.9
R1	109.36	3875	2828.8/730	25.9
R2	102.8	6125	3711.8/606	36.1
R3	156.7	5450	2550.6/468	16.3

NP40	Tissue (mg)	660 assay (µg/ml)	Total (µg/ l)	µg/mg tissue
N1	106.3	1210	750.2/620	7.06
N2	99.45	1330	771.4/580	7.76
N3	109.2	1630	717.2/440	6.57
R1	109.36	1200	648/540	5.93
R2	102.8	1190	747.3/628	7.27
R3	156.7	960	604.8/630	3.86

SDS	Tissue (mg)	660 assay (µg/ml)	Total (µg/µl)	µg/mg tissue
N1	106.3	135	82.4/610	0.78
N2	99.45	120	70.8/590	0.71
N3	109.2	100	56.2/562	0.51
R1	109.36	135	79.7/590	0.73
R2	102.8	145	67.9/468	0.66
R3	156.7	85	39.3/462	0.25

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Table A2. Selected primers, size of amplified product and running conditions for qPCR analysis.

Gene		Sequence (5'->3')	Size (bp)	qPCR Protocol (45 cycles)					
				Denaturation		Annealing		Extension	
				°C	sec.	°C	sec.	°C	sec.
TGF-β1	F	TTCATGAACCCAAGGGCTACC	104	95	10	65	10	72	15
	R	CTGGTTGTACAGAGCCAGGAC							
TGF-β3	F	CTGTGCGTGAATGGCTCTTG	93			60			
	R	TATCCCCGTTGGGCTGAAAG							
RANKL	F	ACGATCAACGCCACAGACAT	89			65			
	R	TTGGAGATCTTGGCCCAACC							
RANK	F	GAAGCACGTCATGGGACTGA	95			60			
	R	CTGGGCAAGTAAGTCTGGGG							
TRAP	F	AACGTCTCGGCACAGATAGC	101			65			
	R	GACACATTGGACCGTGGGAT							
CALCR	F	CGCCTGTGGTGGTATCATGT	142						
	R	CAGGGCCGTGGATGATGTAA							
NFATc1	F	TCGTGGAGAAAGCACCAGAC	91						
	R	TCGACCACCAAGGAATTCGG							
OPG	F	TGTTCTGGAAACAGTGAATCGAC	80						
	R	ACAGCAAACCTGAAGAACGC							
GAPDH	F	CCATCACCATCTTCCAGGAG	346						
	R	ACAGTCTTCTGGGTGGCAGT							

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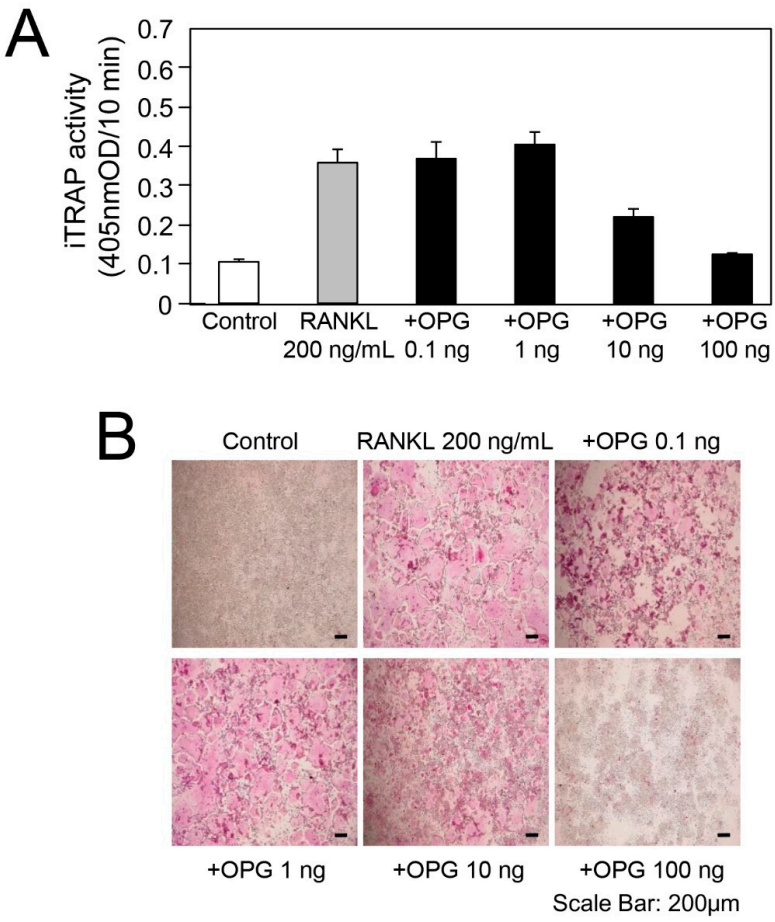
Figure A1. Effect of OPG against RANKL stimulated RAW264 cells. **(A)** iTRAP activity (n = 6 culture wells for each group) and **(B)** TRAP staining of RAW264 cells exposed by RANKL only (200 ng/mL) without (Control) or with the addition of various concentration of OPG (0.1 ng to 100 ng).



Appendix References

1. Matsushima, Y. Growth and differentiation factors exist in porcine periodontal ligament tissues. *J Jpn Soc Periodontol* **2010**, *52*, 24-36.
2. Sasaki, T.; Motegi, N.; Suzuki, H.; Watanabe, C.; Tadokoro, K.; Yanagisawa, T.; Higashi, S. Dentin resorption mediated by odontoclasts in physiological root resorption of human deciduous teeth. *Am. J. Anat.* **1988**, *183*, 303-315.
3. Sasaki, T.; Takahashi, N.; Higashi, S.; Suda, T. Multinucleated cells formed on calcified dentine from mouse bone marrow cells treated with 1α,25-dihydroxyvitamin D3 have ruffled borders and resorb dentine. *Anat. Rec.* **1989**, *224*, 379-391.
4. Addison, W.C. Enzyme histochemical characteristics of human and kitten odontoclasts and kitten osteoclasts: A comparative study using whole cells. *Histochem. J.* **1979**, *11*, 719-735.
5. Oshiro, T.; Shibasaki, Y.; Martin, T.J.; Sasaki, T. Immunolocalization of vacuolar-type H^+-ATPase, cathepsin K, matrix metalloproteinase-9, and receptor activator of NFκB ligand in odontoclasts during physiological root resorption of human deciduous teeth. *Anat. Rec.* **2001**, *264*, 305-311.