

Article

TGF- β and Physiological Root Resorption of Deciduous Teeth

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Abstract: The present study was performed to examine that transforming growth factor beta (TGF- β) in root-surrounding tissues on deciduous teeth during the physiological root resorption regulates the differentiation induction into odontoclast. We prepared root-surrounding tissues with (R) or without (N) physiological root resorption scraped off at three regions (R1-R3 or N1-N3) from the cervical area to the apical area of the tooth and measured both TGF- β and the tartrate-resistant acid phosphatase (TRAP) activities. The TGF- β activity level was increased in N1-N3, whereas the TRAP activity was increased in R2 and R3. *In vitro* experiments for RANKL-mediated osteoclast differentiation revealed that TGF- β in N1-N3 and R1-R3 enhanced the TRAP activity in RAW264 cells. A genetic study indicated that the mRNA level of TGF- β 1 in N1 and N2 was significantly increased, and corresponded with that of osteoprotegerin (OPG). In contrast, the expression level of receptor activator of NF- κ B ligand (RANKL) was increased in R2 and R3. Our findings suggest that TGF- β is closely related to the regulation of OPG induction and RANKL-mediated odontoclast differentiation depending on the timing of RANKL and OPG mRNA expression in the root-surrounding tissues of deciduous teeth during physiological root resorption.

Keywords: cytokine; gene expression; osteoclast; root resorption; pediatric dentistry; protein expression

1. Introduction

Physiological root resorption of human deciduous teeth proceeds from a root non-resorption period to a root resorption period for the formation of permanent teeth. This series of progressions is mediated by odontoclasts, which are produced in the root-surrounding tissues [1]. Odontoclasts possess similar characteristics, such as the ultrastructure, enzymatic and metabolic properties, as osteoclasts [2-5]. During osteoclastogenesis, the receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) and its receptor (RANK) have been shown to play a crucial role in the induction of osteoclast differentiation [6], whereas osteoprotegerin (OPG) is a decoy receptor for RANKL and suppresses osteoclast differentiation [7]. Thus, this mechanism must be taken into consideration, even in odontoclastogenesis.

In periodontal tissue, osteoblasts in the alveolar bone, periodontal ligament (PDL) cells, cementoblasts and gingival epithelial cells express RANKL rather than OPG [8-11]. It has been reported that PDL cells exclusively express OPG under non-resorption conditions, whereas the RANKL expression levels become up-regulated and those of OPG become down-regulated under resorption conditions [12]. Other studies have shown that cytokines released from tooth germ regulate the balance between RANKL and OPG in osteoclasts and osteoclastic bone resorption in alveolar bone [13,14].

Transforming growth factor- β (TGF- β) is a multifunctional cytokine and is critical for cell differentiation. In mammals, TGF- β consists of three isoforms, including TGF- β 1, TGF- β 2 and TGF- β 3, and their structures are highly conserved among isoforms and species [15]. TGF- β 1 negatively regulates osteoclastogenesis via OPG induction with bone marrow stromal cells [16]. Furthermore, TGF- β has been shown to induce OPG expression in osteoblastic cells and inhibit osteoclast differentiation and survival [17]. In contrast, TGF- β exerts contradictory actions on osteoclasts via the regulation of both the proliferation and fusion of osteoclast precursors and the enhancement of RANKL-mediated differentiation through various TGF- β signaling pathways [18–22].

Our aim in this study was to characterize the action of TGF- β in tissues that surround deciduous teeth during physiological root resorption at both the protein and mRNA levels.

2. Results

2.1. Sequential Extraction of Proteins from Root-Surrounding Tissues

We initially attempted to investigate the *in vivo* TGF- β 1 activity in root-surrounding tissues at the protein level. For these experiments, we dissected deciduous teeth with or without root resorption from 5-month-old pigs and scraped off the surrounding tissues on three regions (R1–R3 and N1–N3) at one-centimeter intervals from the cervical areas to the apical area of the tooth (Figure 1A). The scraped surrounding tissues were submitted to a series of three extractions with Triton X, NP-40 and SDS buffers (Figure 1B). These extractions yielded three soluble fractions, designated as Triton X, NP-40, and SDS fractions, which were analyzed via SDS-PAGE stained with Simply Blue Safe Stain and Stains-all (Figure 1C). In all fractions, R3 obtained from the apical area with root resorption was prone to reducing protein bands. The albumin and collagen were mainly collected in the Triton X and NP-40 fractions, respectively. Interestingly, the collagen bands in both the Triton X and NP-40 fractions obtained from R3 were substantially reduced compared with the other fractions; however, the intensity of the albumin band was minimally changed. The Stains-all positive decorin band had an apparent molecular weight of 110 kDa in the SDS fraction, which was reduced in the following order in the deciduous teeth with root resorption: R1 > R2 > R3.

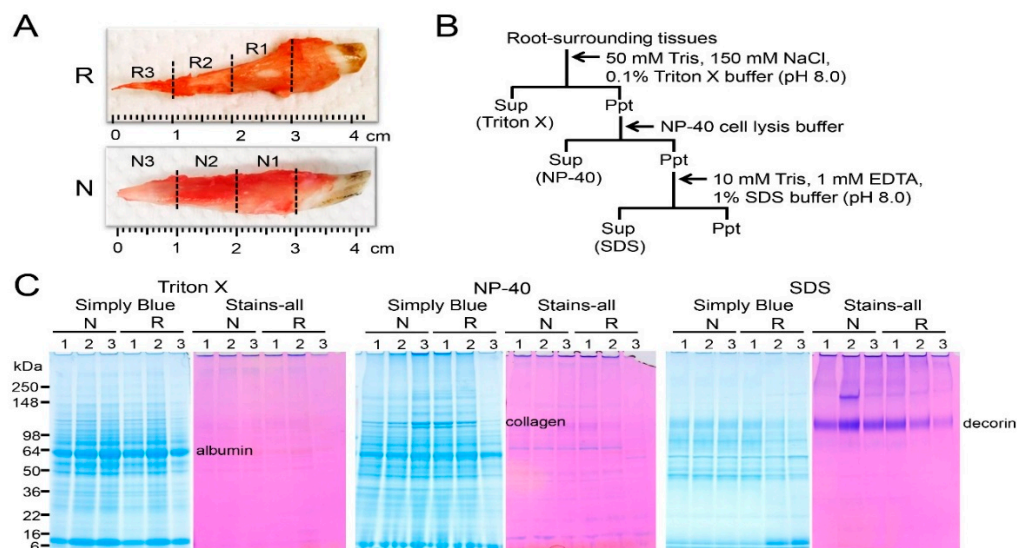


Figure 1. Extraction of proteins in root-surrounding tissues on deciduous teeth. **(A)** Deciduous incisor teeth with (R) or without (N) root resorption from 5-month-old pigs. The root-surrounding tissues were scraped off by dividing them into three regions (R1–R3 and N1–N3) at 1-cm intervals from the cervical area to the apical area of the tooth. **(B)** Flow chart indicates the procedures used to produce extracts for proteins in root-surrounding tissues. Sup: supernatant; Ppt: precipitate. **(C)** SDS-PAGE (5% to 20% gradient gel) stained with Simply Blue Safe Stain (Simply Blue) and Stains-all indicates each fraction.

2.2. Detection of TGF- β and TRAP Activities in Root-Surrounding Tissues and Identification of TGF- β Isoforms

We determined the alkaline phosphatase (ALP)-inducing activity in human periodontal ligament (HPDL) cells enhanced by TGF- β in the Triton X, NP-40 and SDS fractions. The ALP-inducing activity among the three fractions was high in the following order: Triton X > SDS > NP-40 (Figure 2A). In the Triton X fraction, the ALP activity was overall high at all regions in the N group; however, its activity in the R group was reduced in association with root resorption (R1 > R2 > R3). The ALP activity in both the NP-40 and SDS fractions was decreased compared with Triton X. A trace level of ALP activity was detected in all R3 samples. In the Triton X fraction, the enzyme-linked immunosorbent assay (ELISA) against TGF- β 1 antibody showed a detectable signal within the range of 25 to 100 pg per one μ g of sample; however, the assay against TGF- β 2 antibody showed trace levels of detection. The ELISA against TGF- β 3 antibody weakly showed a positive detection in both R1 and R2 within the range of 3.12 to 12.5 pg per one μ g of sample; however, it was minimally detected in the other samples (Figure 2B).

To gain information regarding odontoclasts in each sample, we examined the endogenous TRAP (eTRAP) activity in the Triton X, NP-40 and SDS fractions. The eTRAP activity among the three fractions was high in the following order: Triton X > NP-40 > SDS fractions. In the Triton X fraction, the eTRAP activity in both R2 and R3 were approximately 2.3–3.8-fold higher than R1 (Figure 2C).

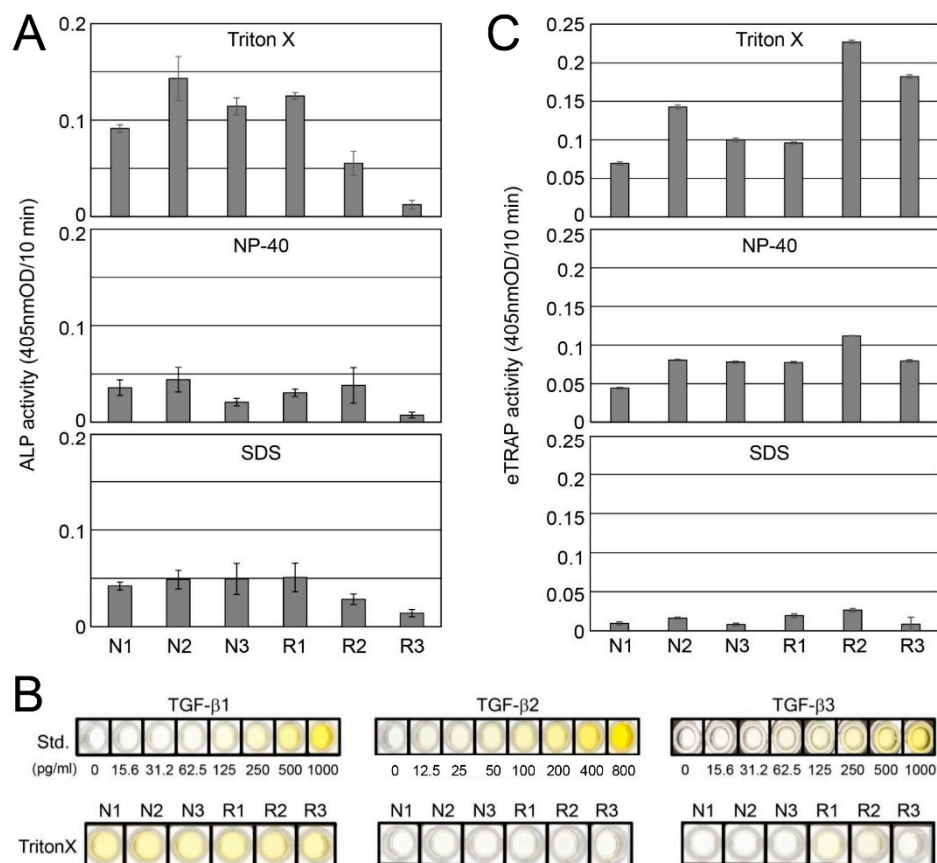


Figure 2. Detection of TGF- β and endogenous TRAP (eTRAP) activities in root-surrounding tissues, and identification of TGF- β isoforms. **(A)** ALP-inducing activity of HPDL cells exposed by protein extracts in N1-N3 and R1-R3 extracted with Triton X, NP-40 and SDS buffers from root surrounding tissues (n = 6 culture wells per sample). **(B)** ELISA for the detection of TGF- β 1, TGF- β 2 and TGF- β 3 in N1-N3 and R1-R3 extracted with Triton X. **(C)** eTRAP activity in N1-N3 and R1-R3 extracted with Triton X, NP-40 and SDS buffers from root surrounding tissues (n = 3 tests per sample).

2.3. *In vitro* Experiment for RANKL-Mediated Osteoclast Differentiation

We confirmed the TGF- β activity in the protein extracts from root-surrounding tissues; thus, we further attempted to perform *in vitro* odontoclast differentiation experiments. In this experiment, we used RAW264 cells, which have been widely used for osteoclast differentiation experiments because odontoclasts possess similar characteristics to osteoclasts [2-5]. We initially confirmed that three rhTGF- β s (rhTGF- β 1, rhTGF- β 2 and rhTGF- β 3) evenly induced RANKL-mediated osteoclast differentiation (Figure 3A), and the addition of OPG suppressed it (Figure A1: Effect of OPG against RANKL stimulated RAW264 cells). The findings regarding TRAP staining for osteoclasts were compared with the controls without RANKL. The addition of each TGF- β isoform enhanced the dyeability of TRAP staining (Figure 3B).

TGF- β activity was highly detected in the Triton X fraction (Figure 2A); thus, we used it for the osteoclast differentiation experiments. The induced TRAP (iTRAP) activity in the RANKL-stimulated RAW264 cells without the addition of N1-N3 and R1-R3 of the Triton X fraction was increased approximately 5.0-fold compared with the RANKL-unstimulated RAW264 cells. The addition of each Triton X fraction enhanced the iTRAP activity level in the N group (approximately 1.2 ~1.3-fold) and the R group (approximately 1.08~1.1-fold) against the level of RANKL only as a baseline (Figure 3C). Interestingly, the level of increased iTRAP activity nearly corresponded to the ALP-inducing activity in the Triton X fraction.

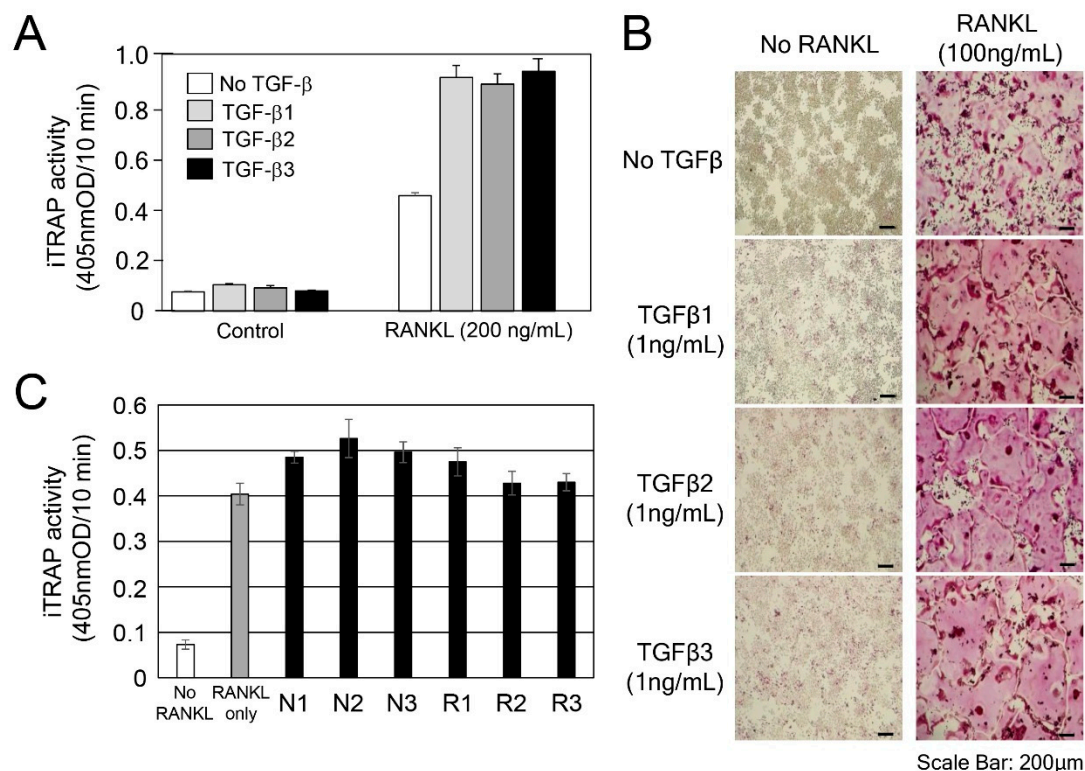


Figure 3. *In vitro* experiment for RANKL-mediated osteoclast differentiation. **(A)** Induced TRAP (iTRAP) activity of RAW264 cells exposed by TGF- β isoforms (1 ng/mL each) with (RANKL) or without (Control) the addition of RANKL (200 ng/mL) (n = 6 culture wells per group). **(B)** TRAP staining for RAW264 cells exposed by TGF- β isoforms without (left panel) or with (right panel) RANKL (100 ng/mL). **(C)** iTRAP activity of RAW264 cells exposed by protein extracts in N1-N3 and R1-R3 of root surrounding tissues with or without the addition of GST-RANKL (200 ng/mL).

2.4. Gene Expression in Root-Surrounding Tissues

We identified both TGF- β 1 and TGF- β 3 in the Triton X fraction (Figure 2B); thus, we further attempted to investigate the genetic information of odontoclast differentiation in root-surrounding

tissues. We performed quantitative real-time PCR (qPCR) assays with TGF- β 1 and TGF- β 3, RANKL, RANK, TRAP/ACP5, calcitonin receptor (CALCR) and nuclear factor of activated T-cells 1 (NFATc1) representing odontoclast promotion and OPG representing odontoclast suppression (Figure 4). To obtain quantitative information, we normalized the quantity of the root-surrounding tissue per sample in each amplification with GAPDH using a standard mathematical model for relative quantification in a qPCR system; this approach assumed a comparable proportion of odontoclasts in each sample. The expression levels of TGF- β 1 mRNA in N1 and N2 were increased (approximately 3.4~3.9-fold) compared with N3, whereas the TGF- β 1 mRNA level in R1 was increased (approximately 2.5~3.3-fold) compared with R2 and R3. The mRNA level of TGF- β 3 in the N group exhibited the opposite of the pattern and the TGF- β 1. Its level was increased in N3 compared with N1 and N2 (approximately 1.5~2.0-fold); however, the mRNA level of TGF- β 3 in the R group was similar to TGF- β 1.

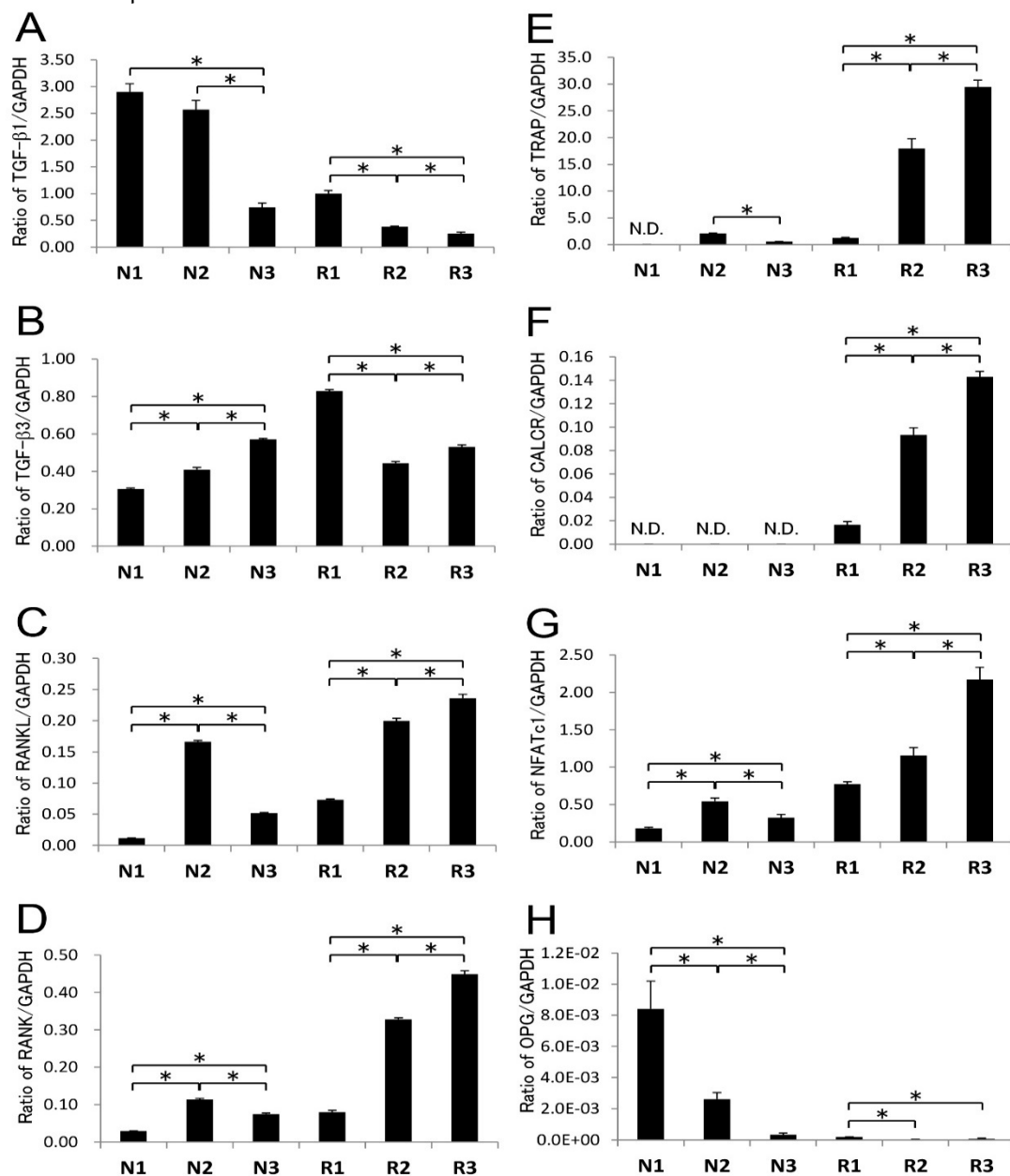


Figure 4. Gene expression in root surrounding tissues. mRNA expression via qPCR analysis of (A) TGF- β 1, (B) TGF- β 3, (C) RANKL, (D) RANK, (E) TRAP, (F) CALCR, (G) NFATc1 and (H) OPG. Each ratio was normalized to the relative quantification data of each mRNA in N1-N3 and R1-R3 of root-surrounding tissues compared with a reference gene (GAPDH), which was generated on the basis of a mathematical model for relative quantification in a qPCR system (n = 6 tissues).

The mRNA levels of RANKL in R2 and R3 were increased (approximately 2.9~3.4-fold) compared with R1, and the level increased with the progression of root resorption. The expression pattern of RANKL mRNA in the R group was approximately the same as the other osteoclast marker genes, such as RANK, TRAP, CALCR and NFATc1. The RANKL mRNA level in N2 was also increased compared with N1 and N3 (approximately 3.5~8.8-fold). The mRNA levels of OPG in N1 and N2 were significantly increased (approximately 12~40-fold) compared with N3. Only a trace expression of OPG was identified in the R group. Based on our qPCR analysis, we calculated the RANKL/OPG ratio; the ratio of N1: N2: N3 was 1.34: 63.8: 157, whereas the ratio R1: R2: R3 was 393: 5192: 3092.

3. Discussion

We summarized the relationships of TGF- β , protein components and odontoclastogenesis-associated factors in the root-surrounding tissues of deciduous teeth during the physiological root resorption in Table 1.

Table 1. Relationships among TGF- β , protein components and odontoclastogenesis-associated factors in the root-surrounding tissues of deciduous teeth during physiological root resorption.

Root-Surrounding Tissue		Non-Resorption			Resorption		
		Cervical	Center	Apica l	Cervica l	Center	Apical
Region		N1	N2	N3	R1	R2	R3
Protein Study	Collagen	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑	↑ ↑	trace
	Decorin	↑	↑ ↑ ↑	↑ ↑	↑ ↑	↑	trace
	ALP activity	↑	↑ ↑	↑ ↑	↑ ↑	↑	trace
	eTRAP activity	↑	↑	↑ ↑	↑ ↑	↑ ↑ ↑ ↑	↑ ↑ ↑
In vitro Study	iTRAP activity	↑	↑ ↑	↑	↑	trace	trace
Genetic Study (mRNA level)	TGF- β 1	↑ ↑ ↑ ↑	↑ ↑ ↑ ↑	↑ ↑	↑ ↑	↑	↑
	TGF- β 3	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑	↑	↑ ↑
	RANKL	trace	↑ ↑ ↑	↑	↑	↑ ↑ ↑	↑ ↑ ↑ ↑
	RANK	trace	↑ ↑	↑	↑	↑ ↑ ↑	↑ ↑ ↑ ↑
	TRAP	N.D	↑	trace	↑	↑ ↑ ↑	↑ ↑ ↑ ↑
	CALCR	N.D	N.D	N.D	↑	↑ ↑ ↑	↑ ↑ ↑ ↑
	NFATc1	trace	↑ ↑	↑	↑ ↑	↑ ↑ ↑	↑ ↑ ↑ ↑
	OPG	↑ ↑ ↑ ↑	↑ ↑	trace	trace	trace	trace
	RANKL/OPG ratio	1.34	63.8	157	393	5192	3092

N.D : not determined

3.1. TGF- β in Root-Surrounding Tissues of Deciduous Teeth

Decorin binds to TGF- β and represents the candidate for the sequestration of TGF- β reservoirs [23-26]. Decorin contains independent binding sites for TGF- β and type I collagen [27]. We demonstrated that TGF- β , collagen and decorin were independently extracted in the Triton X, NP-40 and SDS fractions, and the SDS-PAGE patterns of both collagen and decorin were reduced with the progression of root resorption (i.e., R1 > R2 > R3). Based on the finding that the ALP-inducing activity in the Triton X fraction also decreased with the progression of root resorption, our data suggest that both collagen and decorin provide a retention capability for TGF- β activity; however, TGF- β may have dissociated from them during extraction.

3.2. *In vitro* Experiment for RANKL-Mediated Osteoclast Differentiation

In mammals, three TGF- β isoforms (TGF- β 1 to - β 3) have been identified and their structures are highly conserved among isoforms and species, with each isoform exhibiting similar functions *in vitro* [28,29]. However, a recent study showed that treatment with adrenaline enhanced the mRNA expression of only TGF- β 3 and not TGF- β 1 or TGF- β 2 in RAW264 cells [30]. This isoform-specific regulation of TGF- β mRNA expression in RAW264 cells led us to consider the effects of the three TGF- β isoforms when RAW264 cells were implemented in the present study. Therefore, we initially confirmed that three recombinant human TGF- β s (rhTGF- β 1, rhTGF- β 2 and rhTGF- β 3) evenly induced RANKL-mediated osteoclast differentiation, and the addition of OPG suppressed it. Based on these findings, our *in vitro* experiments for RANKL-mediated osteoclast differentiation indicated that the addition of each sample in the Triton X fraction into RAW264 cells enhanced the iTRAP activity, which corresponded to the ALP-inducing activity level in each sample. These findings demonstrate that TGF- β s in Triton the X fraction induced RANKL-mediated osteoclast differentiation *in vitro*.

3.3. Potential Roles of TGF- β in Root-Surrounding Tissues of Deciduous Teeth

In periodontal tissue, RANKL has been expressed in not only osteoblasts in the alveolar bone but also periodontal ligament cells, cementoblasts and gingival epithelial cells [8-10]. To enhance RANKL-mediated osteoclast differentiation, TGF- β induces RANKL mRNA expression and activates RANKL promoter activity [31,32]. In the root resorption period, we revealed that the mRNA level of RANKL was high in R2 and R3, whereas the mRNA level of TGF- β 1 and TGF- β 3 and the ALP-inducing activity in the Triton X fraction were high in R1. We also demonstrated that the mRNA levels of osteoclast marker genes, such as RANK, CALCR, TRAP and NFATc1, were substantially increased in R2 and R3, as well as the eTRAP activity in the Triton X fraction. Taken together, our findings suggest that TGF- β 1 and TGF- β 3 may contribute to the induction of RANKL mRNA expression in R1, followed by RANKL-mediated osteoclast differentiation in R2 and R3.

During the root resorption period, cytokines other than TGF- β s, such as interleukin-1, interleukin-33 and parathyroid hormone-related peptide, released from the tooth germ have been associated with the promotion of RANKL expression [13,14,33]. Despite the high levels of RANKL mRNA expression in R2 and R3 (approximately 8.0- to 13-fold higher than R1 as the RANKL/OPG ratio), we revealed that the mRNA levels of TGF- β 1 and TGF- β 3 exhibited a decreasing tendency in R2 and R3, as well as the ALP-inducing activity in the Triton X fraction. These findings suggest that TGF- β s may not be the main factor; in contrast, other cytokines or bone resorption factors may enhance RANKL mRNA expression.

In addition to RANKL-mediated osteoclast differentiation, TGF- β has been shown to induce OPG mRNA expression in osteoblastic cells and inhibit osteoclast differentiation; however, the effect of TGF- β 1 on the expression of OPG mRNA was transient [16,17]. In a molecular basis study, TGF- β directly affected the transcriptional activity of the OPG gene, which generates the reciprocal up-regulation of OPG mRNA and the down-regulation of RANKL mRNA [11]. In the root non-resorption period, we demonstrated that the mRNA levels of both TGF- β 1 and OPG were significantly increased in N1, and the ALP-inducing activity in the Triton X fraction was identified at

the modest level; however, its level was decreased compared with N2 and N3. In addition, a trace level of RANKL mRNA expression was identified in N1. The *in vitro* experiments demonstrated that TGF- β s induce RANKL-mediated osteoclast differentiation; however, our findings suggest that TGF- β 1 may regulate the expression of OPG mRNA rather than RANKL in N1. We also demonstrated that the mRNA levels of RANKL and TGF- β 1 were significantly increased in N2. In addition, the ALP-inducing activity in the Triton X fraction indicated it was at the highest level of all samples. Moreover, we revealed that the mRNA levels of RANK and TRAP exhibited an increased tendency in N2; however, the eTRAP activity was approximately equal between N1 and N2 in the Triton X fraction. Considering that the OPG mRNA expression was identified in N2 and the RANKL/OPG ratio in N2 increased approximately 48-fold compared with N1, our data suggest that TGF- β 1 may regulate both OPG mRNA expression and RANKL-mediated osteoclast differentiation in N2; however, the OPG protein in N1 and N2 may suppress RANKL-mediated osteoclast differentiation. The mRNA levels of TGF- β 1, OPG, RANK, RANKL and TRAP were decreased in N3; however, the expression level of TGF- β 3 mRNA indicated an increasing tendency. Moreover, the ALP-inducing activity in the Triton X fraction was retained. We demonstrated that the RANKL/OPG ratio in N3 was approximately 2.5-fold higher than N2, and the eTRAP activity in the Triton X fraction in N3 was slightly increased compared with N1 and N2. These findings suggest that the stage of N3 may gradually switch from OPG mRNA expression to RANKL-mediated osteoclast differentiation.

The RANKL/OPG ratio has been used as an indicator of bone resorption, and it represents an important determinant of bone mass and skeletal integrity, such as in fracture healing [34]. We demonstrated that the RANKL/OPG ratio increased with the progression of root resorption (i.e., N1 < N2 < N3 and R1 < R2 < R3). This finding suggests that OPG is transiently expressed during the root non-resorption period, whereas RANKL is rapidly expressed with the progression of root resorption.

The present study demonstrated that TGF- β is closely related to the regulation of both OPG induction and RANKL-mediated osteoclast differentiation, depending on the timing of the mRNA expression of RANKL and OPG factors in the root-surrounding tissues of deciduous teeth during physiological root resorption. Studies are required to identify the inducing substances for regulating and switching this balance.

4. Materials and Methods

All experimental procedures that involved the use of animals were reviewed and approved by the Ethics Committee of the Institute of Tsurumi University, Yokohama, Japan.

4.1. Tissue Preparation

Tooth germs of permanent incisors were surgically extracted from the mandibles of five-month-old pigs obtained from the Meat Market of Metropolitan Central Wholesale Market (Tokyo, Japan). Deciduous teeth with (R) or without (N) root resorption were surgically extracted and the surrounding tissues were scraped off at one-centimeter intervals from three regions (R1-R3; approximately 103-157 mg each and N1-N3; approximately 99-109 mg each) from the tooth's cervical area to the apical area (Figure 1A). Each sample was utilized immediately for RNA isolation or after storage at -80 °C for protein extraction.

4.2. Sequential Extraction of Proteins from Root-Surrounding Tissues

The sequential extraction of proteins from the R1-R3 and N1-N3 samples was conducted in the following order: 50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X buffer (pH 8.0) (Triton X), NP-40 cell lysis buffer (Thermo Fisher Scientific., Waltham, MA, USA) (NP-40) and 10 mM Tris-HCl, 1 mM EDTA, and 1% SDS buffer (pH 8.0) (SDS). The collected tissue weights from each group, the total micrograms of protein per sample, and the micrograms of protein extracted per milligram of tissue are provided in 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.

4.3. Alkaline Phosphatase (ALP) Activity Assay (ALP-HPDL System)

Each sample (10 µg) fractionated with Triton X, NP-40 and SDS was added into human periodontal ligament fibroblast (HPDL) cells (LONZA, Walkersville, MD, USA), and the cell culture and ALP activity were assessed using our previous method [35].

4.4. SDS-PAGE

SDS-PAGE was performed with 5% to 20% e-PAGEL mini gel (ATTO Corporation, Tokyo, Japan). The gels were stained with Simply Blue Safe Stain (Life Technologies/Invitrogen, Carlsbad, CA, USA) or Stains-all (Sigma-Aldrich, St. Louis, MO, USA).

4.5. Endogenous and Induced Tartrate-Resistant Acid Phosphatase (TRAP) Assays and TRAP Staining

For the endogenous TRAP assay, each sample (10 µg) in Triton X, NP-40 and SDS fractions was directly incubated with 0.1 M *p*-nitrophenylphosphate as a substrate in TRAP buffer. The induced TRAP assay and TRAP staining were performed using our previous method [36]. Each sample (10 µg) in Triton X was added to RAW264 cells with or without GST-RANKL (200 ng/mL).

4.6. Enzyme-Linked Immunosorbent Assay (ELISA)

The identification of the TGF-β isoforms in the N1-N3 and R1-R3 samples was determined by a sandwich enzyme immunoassay method using a Quantikine ELISA kit (R&D Systems) for TGF-β1 and TGF-β2 and an ELISA Cloud-Immunoassay kit (Cloud-Clone Corp., Houston, TX, USA) for TGF-β3. The ELISA for N1-N3 and R1-R3 in the Triton X fraction (4 µg each for TGF-β1 and TGF-β2 and 0.4 µg each for TGF-β3) was performed following the manufacturer's instructions.

4.7. Quantitative Real-Time PCR (qPCR)

The root-surrounding tissues at the R1-R3 and N1-N3 regions were extracted with RNA extraction reagent (Isogen, Nippon Gene Co., Ltd., Tokyo, Japan). Purified total RNA (2 µg) was reverse transcribed, and qPCR was subsequently performed using the SYBR Green technique on a LightCycler Nano (Roche Diagnostics, Mannheim, Germany). The selected primers, running conditions and sizes of the amplified product are provided in Table A2: Selected primers, size of amplified product and running conditions for qPCR analysis. Each ratio was normalized to the relative quantification data of TGF-β1, TGF-β3, RANKL, RANK, TRAP, calcitonin receptor (CALCR), nuclear factor of activated T-cells 1 (NFATc1) and OPG compared with a reference gene (GAPDH), which was generated on the basis of a mathematical model for relative quantification in the qPCR system.

4.8. Statistical analysis

For the qPCR analysis, in an enzyme assay with the ALP-HPDL system and TRAP assay, all values were represented as the means ± standard error (s.e.m.). Statistical significance (*) was determined using unpaired Student's *t*-tests. In all cases, *p* < 0.05 was regarded as statistically significant.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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Author Contributions: Conceived and designed the experiments: Emi Shimazaki, Makoto Fukae, Yasuo Yamakoshi and Yoshinobu Asada. Performed the experiments: Emi Shimazaki, Takeo Karakida, Ryuji Yamamoto, Saeko Kobayashi and Yasuo Yamakoshi. Analyzed the data: Takeo Karakida, Ryuji Yamamoto and Yasuo Yamakoshi. Wrote the paper: Emi Shimazaki, Yasuo Yamakoshi and Yoshinobu Asada.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
NP-40	nonidet P-40
kDa	kilodalton
ACP5	acid phosphatase 5
PCR	polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione S-transferase

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