In Vitro Guidance of Dental Pulp Cells by Nd:YAG Laser-Irradiated Endothelial Cells

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Abstract

Objective: After endothelial cells were ablated by neodymium:yttrium-aluminum-garnet (Nd:YAG) laser irradiation, we investigated the response of pulp cells by examining the expression of transforming growth factor beta-1 (TGF- β 1). **Background data:** The reaction of stimulated blood vessels is related to the initiation of dentinogenesis. After artificial injury of endothelial cells, pulp cells migrate to the site of the injured endothelial cells. **Materials and methods:** Rat aortic endothelial cells were cultured in the lower compartment of the experimental assembly, and a pulsed Nd:YAG laser was used to ablate these cells. Pulp cells were fluorescence labeled and cultured in the upper compartment. After 7–14 days of laser irradiation, total RNA was extracted from the cells in the lower chamber, and RT-PCR was performed to examine the expression of TGF- β 1 and osteocalcin mRNA. TGF- β 1 was also examined with immunohistochemistry. **Results:** Seven days after laser irradiation, migrating pulp cells that expressed TGF- β 1 were observed in the lower compartment, and the expression of TGF- β 1 mRNA and osteocalcin mRNA was altered. Without laser irradiation, few migrating pulp cells were observed, and the expression of TGF- β 1 mRNA and osteocalcin mRNA was weak. These results suggested that TGF- β 1 mRNA expression is detected earlier in pulp cells rather than in endothelial cells following injury to endothelial cells. **Conclusions:** Using the Nd:YAG laser as an ablative stimulant, this study model was useful for investigating pulp–endothelial cell interactions in reparative dentinogenesis.

Introduction

THE DENTAL PULP IS A MICROCIRCULATORY SYSTEM that lacks true arteries and veins. The pulpal microcirculation is a dynamic system that regulates blood flow in response to nearby metabolic events including dentinogenesis.¹ Pulp irritation following dental procedures, such as cavity preparation, results in enlargement of the blood vessels in dental pulp. Perivascular stem cells proliferate in response to such irritations and are strong enough to cause odontoblast injury.² For example, caries induce the release of dentin matrix components that contribute to angiogenic events that support pulp regeneration.³ The response of stimulated blood vessels contributes to the initiation of dentinogenesis. Mathieu et al. reported that pulp cells migrate to the site of injured endothelial cells.⁴ Endothelial injury is involved in the recruitment of odontoblast-like cells to the damaged site, and perivascular stem cells can proliferate in response to odontoblast injury.²

Transforming growth factor- β 1 (TGF- β 1) has been implicated in tissue repair and regeneration after injury.⁵ Lin et al. reported that TGF- β 1 may play a role in odontoblast differ-

entiation and in reparative dentinogenesis in human dental pulp.⁶ Higher expression of TGF- β 1 is found in the odontoblastic and subodontoblastic layers of irreversible pulpitis specimens, indicating its role in dentinal repair after control of pulp inflammation.⁶

We consider that artificial injury to endothelial cells reflects the reaction of stimulated vessels in the pulp, similar to what occurs in pulpitis. Therefore, the objective of this study was to investigate the response of pulp cells by examining the expression of TGF- β 1 after endothelial cells were artificially injured using neodymium:yttrium-aluminum-garnet (Nd:YAG) laser irradiation.

Materials and Methods

This study was approved by Showa University Animal Care and Ethical Use Committee (No: 11064).

Animals

Five-week-old Wistar strain male littermate rats were used to establish the cell culture. Five-week-old rats were used

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because of the ease of anesthetization and removal of mandibles and pulps. The methods that established the culture system for rat pulp cells were used as reference.⁷

Green fluorescent protein vector transfection

Pulp cells were fluorescence labeled by transfecting with the pAcGFP1-Actin vector, which encodes enhanced green fluorescent protein (Clontech, Palo Alto, CA). Pulp cells were grown in a 75-cm² flask for 7 days until they reached subconfluency, and were then transfected with 1 μ g pAcGFP1-Actin vector using the TransIT[®]-LT1 transfection reagent (Mirus Bio. Co., Ltd., Madison, WI). After 48 h, the pulp cells were removed.

Cell culture

Pulp cell culture was performed as described previously.⁷ Five independent primary cultures were performed for each experiment. The insert cell culture system (Corning, Acton, MA) containing a polycarbonate membrane (8 μ m pore size) was used to study cell migration in response to injury.⁴ After 9 days, the fluorescence-labeled pulp cells were removed from the 75-cm² flask and cultured in the upper compartments of six-well plates at a density of 10⁴ cells/cm² in α -MEM medium (GIBCO; Invitrogen, Grand Island, NY) with 10% heat-inactivated calf serum. Rat aortic endothelial cells (Cell Applications, INC., San Diego, CA) were cultured in rat endothelial cell growth medium with growth supplements (Cell Applications, INC.) in the lower compartment. Controls were performed without pulp cells in the upper compartment.

Laser irradiation

After the endothelial cells reached subconfluency, a pulsed Nd:YAG laser (d-Lase 300; American Dental Laser, Birmingham, MI) was used. This laser emits at a wavelength of 1.064 μ m and has a flexible fiber delivery system (ϕ =0.32 mm). Laser irradiation was performed with a focused beam at a pulse energy of 100 mJ and output power of 0.5 W with 5 pulses/sec for 30 sec at a distance of 3 mm from the bottom of the six-well plates five times per well with the endothelial cells in the lower compartment (Fig. 1). Controls were performed without laser irradiation.

Total RNA preparation

Total RNA was extracted from the cells in the lower compartment on days 7 and 14 after laser irradiation using an RNaid kit (BIO 101, Inc., La Jolla, CA). Total RNA (1 μ g)

FIG. 1. Schematic of the experimental setup including endothelial cells in the lower compartment, the fiber tip of the pulsed Nd:YAG laser, and the laser-irradiated spots in the well.

was converted to cDNA using reverse transcriptase (High Capacity RNA-to-cDNA Master Mix; Applied Biosystems Inc., Tokyo, Japan).

RT-PCR

PCR amplification was performed in a 50- μ l PCR reaction mixture containing target cDNA, 10 mM dNTPs, 0.25 μ l Ex-Taq DNA polymerase (5 U/ μ l) (Takara Ex Taq[®]; Takara Bio Inc., Shiga, Japan), and 10 pmol of each specific primer set. The nucleotide sequences of the primer pairs for TGF- β 1 are 5'-GTGCTAATGGTGGACCGCAAC-3' (sense) and 5'-TCCCGAATGTCTGACGTATTGAAG-3' (antisense), for osteocalcin are 5'-AGACTCCGGCGCTACCTCAA-3' (sense) and 5'-CGTCCTGGAAGCCAATGTG-3' (antisense), and for GAPDH are 5'-GACAACCTTTGGCATCGTGG-3' (sense) and 5'-ATGCAGGGATGATGTTCTGG-3' (antisense).

The reaction was amplified for 35 cycles, with denaturation at 94°C for 30 sec, annealing at 64°C (TGF- β 1 and osteocalcin) or 63°C (GAPDH) for 30 sec, and extension at 72°C for 90 sec. The PCR products were visualized on a 2% agarose gel.

Immunohistochemistry

Immunohistochemistry was performed 7 and 14 days after laser irradiation. The endothelial cells were fixed for 15 min in 10% neutral-buffered formalin and then incubated with antibodies for 30 min at room temperature. Expression of TGF- β 1 was detected using a rabbit polyclonal antibody against recombinant human TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500). Block Ace[®] (50% dilution; DS Pharma Biomedical, Osaka, Japan) was used to dilute the antibody, and PBS (pH 7.4) was used for rinsing. The cells were then incubated with cyanine 3.29-OSu Cy3-conjugated goat anti-rabbit IgG (Abcam plc. Cambridge, UK; 1:500) for 15 min at room temperature. After rinsing, the cells were examined using a confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

Results

RT-PCR

Figure 2 shows the expression of TGF- β 1 and osteocalcin in the endothelial cells in the lower compartment of six-well plates in the presence or absence of pulp cells in the upper compartment. Seven days after laser irradiation, the expression of TGF- β 1 mRNA and osteocalcin mRNA was distinct compared to expression in endothelial cells in the absence of pulp cells (Fig. 2a, b). In the absence of pulp cells, the expression of TGF- β 1 mRNA was comparatively weak, and osteocalcin mRNA expression was not detected (Fig. 2b). Without laser irradiation, the expression of TGF- β 1 mRNA and osteocalcin mRNA was weak (Fig. 2c). Fourteen days after laser irradiation, expression of TGF- β 1 mRNA and osteocalcin mRNA was observed in the presence and absence of pulp cells (Fig. 2e, f). Thereafter, the expression patterns with pulp cells were similar to those without pulp cells. The expression of TGF- β 1 mRNA and osteocalcin mRNA was decreased compared with expression at 7 days with pulp cells. The expression of TGF- β 1 mRNA and osteocalcin mRNA was slightly increased compared with expression at 7 days without pulp cells (Fig. 2f). Without laser irradiation, the expression of TGF- β 1 mRNA and osteocalcin mRNA was weak (Fig. 2g). In

FIG. 2. RT-PCR analysis of TGF- β 1 and osteocalcin mRNA expression in the lower compartment. Seven (a–d) and 14 (e–h) days after laser irradiation. pel: pulp cells in the upper compartment (pulp [+]), laser irradiation to the endothelial cells in the lower compartment (laser [+]); el: pulp (–), laser (+); pe: pulp (+), laser (–); e: pulp (–), laser (–). G, GAPDH (133 bp); T, TGF- β 1 (95 bp); B, osteocalcin (133 bp).

the absence of pulp cells and laser irradiation, the expression of these mRNAs was not observed (Fig. 2d, h).

Immunocytochemistry

Figure 3 shows immunocytochemical localization of TGF- β 1 and the migration of green fluorescent protein-labeled pulp cells. Green pulp cells were observed in the upper

compartment (p). Seven days after laser irradiation and in the presence of pulp cells in the upper compartment, a few migrated green pulp cells were observed among the endothelial cells in the lower compartment (Fig. 3A, pel, a). TGF- β 1 staining was red (Fig. 3A, pel, b). Double-stained cells were orange, which represented green pulp cells that had migrated and expressed TGF- β 1 (Fig. 3A, pel, c).

After laser irradiation and in the absence of pulp cells in the upper compartment, TGF- β 1 staining was very weak or absent (Fig. 3A, el). Without laser irradiation and in the presence of pulp cells in the upper compartment, TGF- β 1 staining was very weak (Fig. 3A, pe). In the absence of both pulp cells and laser irradiation, TGF- β 1 staining was not observed (Fig. 3A, e). Fourteen days after laser irradiation and in the presence of pulp cells, the numbers of migrated, green pulp cells were greater among the endothelial cells in the lower compartment (Fig. 3B, pel, a). TGF- β 1 staining was observed in many cells (Fig. 3B, pel, b). Some TGF- β 1-stained cells in the lower compartment were orange (Fig. 3B, pel, c). After laser irradiation and in the absence of pulp cells, a few migrated, green pulp cells were observed in the lower compartment, and they expressed TGF- β 1 (Fig. 3B, el). Without laser irradiation and in the presence of pulp cells, many TGF- β 1-stained cells were present in the lower compartment (Fig. 3B, pe, b). Some were pulp cells, which were stained orange.

In the absence of both pulp cells and laser irradiation, TGF- β 1 staining was not observed (Fig. 3, e).

Discussion

Angiogenesis is very important for regeneration of the dentin-pulp complex, and signaling of angiogenic events at

FIG. 3. Confocal laser scanning microscopic images of immunocytochemical labeling of dental pulp and endothelial cells in the lower compartment after laser irradiation. (A) 7 days; (B) 14 days; pel: pulp (+), laser (+); el: pulp (–), laser (+); pe: pulp (+), laser (–); e: pulp (–), laser (–); p: fluorescence-labeled pulp cells in the upper compartment. a, migrated green fluorescence-labeled pulp cells; b, immunocytochemical labeling of TGF- β 1; c, colocalization of both green fluorescent protein and TGF- β 1 (orange).

sites of injury is critical for tissue repair. "Supportive" cells, including endothelial cells, are required for elaborate dentin-pulp engineering, and the co-culture model of pulp and endothelial cells highlights the role of interactions between these cells for dentin regeneration.⁸ Mathieu et al. reported that pulp progenitor cells migrate in response to endothelial cell injury in this study model.⁴ They used sterile scalpels to injure the endothelial cells. Instead of scalpels, we used Nd:YAG laser irradiation to injure the endothelial cells. Nd:YAG laser irradiation is an effective stimulus for many kinds of cells.⁹ We performed sterilized manipulation, and the energy level was changed as needed.

Seven days after laser irradiation and in the presence of pulp cells, migrated, green pulp cells that expressed TGF- β 1 were observed among the endothelial cells in the lower compartment. After laser irradiation and in the absence of pulp cells, TGF- β 1 staining was very weak or absent.

Seven days after laser irradiation in the presence of pulp cells, TGF- β 1 mRNA and osteocalcin mRNA expression was distinct compared to expression in endothelial cells in the absence of pulp cells. We previously examined gene expression during the reparative dentin process and the patterns of serial changes in the relative quantity of TGF- β 1 and osteocalcin mRNA during odontoblast-like cell differentiation in rat cultured pulp cells.¹⁰ The expression of TGF- β 1 mRNA increased by day 15 before osteocalcin mRNA expression was upregulated. TGF- β 1 mRNA gradually decreased by day 28 when cells were cultured in mineralized medium. The expression of osteocalcin mRNA was observed later at 15 days when it was very weak, but expression rapidly increased by day 28. Osteocalcin mRNA is expressed at high levels in fully mature odontoblasts during dentinogenesis.¹¹ In our current study, the expression of osteocalcin mRNA was examined in addition to TGF- β 1 mRNA.

In our current study, 7 days after laser irradiation, expression of osteocalcin mRNA was detected in endothelial cells when pulp cells migrated, but in the absence of pulp cells, the expression of osteocalcin mRNA was not observed. These results suggest that migrated pulp cells may be pulp progenitor cells that differentiated into fully mature odontoblasts that expressed osteocalcin mRNA and TGF- β 1 mRNA. However, fully mature odontoblasts would not be expected to have differentiated after only 7 days of subculture. Tcls et al. reported that after a 2-week culture, labeled cells had migrated to the injury site and secreted reparative dentin.^{2, 12} Nd:YAG laser irradiation may contribute to accelerated pulp cell differentiation.

Other investigators have studied pulp progenitor cells and vascularized tissues. Injured endothelial cells release signaling molecules that initiate the inflammatory reaction and the healing process,¹³ and pulp progenitor cells can migrate in response to endothelial cell injury.⁸ In highly vascularized tissues, after transplantation of progenitor cells, tubular dentin production is observed.^{14,15} In highly vascularized tissues such as those containing endothelial cells, differentiation of pulp progenitor cells may be accelerated, and the response of endothelial cells to Nd:YAG laser irradiation may be larger than the response *in vivo*. Laser-irradiated endothelial cells may express multiple molecules and factors that contribute to pulp cell differentiation. Effective dentin formation following laser irradiation of exposed pulp has been studied.¹⁶ By using laser irradiation to stimulate

endothelial cells, it may be possible to accelerate reparative dentin formation clinically. Further investigations are needed.

We consider that at an early time (7 days), pulp cells distinctly expressed both mRNAs after migrating to the site of laser-irradiated endothelial cells or co-cultured pulp-endothelial cells. Fourteen days after laser irradiation and even in the absence of pulp cells, laser-irradiated endothelial cells expressed both mRNAs. TGF- β 1 staining was observed in endothelial cells. These results suggested that after injury of endothelial cells, TGF- β 1 mRNA expression was detected earlier in pulp cells and later in endothelial cells.

In conclusion, using the Nd:YAG laser as a stimulus, this study model was useful for investigating pulp-endothelial cell interactions during reparative dentinogenesis, which was the result of many factors, such as laser irradiation and expression of TGF- β 1.

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Author Disclosure Statement

No conflicting financial interests exist.

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