Early gene expression analyzed by a genome microarray and real-time PCR in osteoblasts cultured with a 4-META/MMA-TBB adhesive resin sealer

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Objectives. Adhesive resin sealer systems have also been applied for endodontics to seal the root canal system. This study was designed to confirm the mechanism of intracellular molecular events *in vitro* cell culture system with a 4-META resin sealer.

Study design. The gene expression patterns relating to cell growth and differentiation were examined using a human genome expression microarray and real-time PCR analyses in hard tissue forming osteoblasts cultured with and without a 4-META resin sealer.

Results. There was no significant difference in the cell number between the control and adhesive sealer groups. An increased expression of integrin β, TGF-β related protein, craniofacial development protein 1, and PI3K genes was demonstrated. The integrin β and PI3K genes showed extremely high ratios.

Conclusions. The signal transduction pathway, at least through the PI3K/Akt cascade for cell proliferation and differentiation, can be controlled by some components of this type of adhesive resin sealer.

The purpose of root canal treatment is to prevent and/or to treat pulp and periapical pathoses.[1] Irrigation and medication usually yield sufficient infection control of the root canal system. Secondary infection is generally prevented by the final sealing of the root canal with various types of filling materials. Therefore, root canal filling to seal the root canal system from the outside environment is the most important step in ensuring a good prognosis.

Traditional root filling material was composed of a gutta-percha core. However, gutta-percha characteristically demonstrates a high coefficient of thermal expansion and shrinkage. Furthermore, it can not adhere to root dentine nor does it possess any sealing properties. This means that a sealer cement containing zinc oxide and eugenol, calcium hydroxide, or epoxy resin is required to provide resistance to bacteria after root canal filling.[2-5] Furthermore, resin systems adhesive to dentine have also been applied for endodontics. These adhesion technologies were originally borrowed from restorative dentistry. A 4-methacryloxyethyl trimellitate anhydride/ methacrylate- tri-*n*-butyl borane (4-META/MMA-TBB) adhesive (4-META) resin developed in Japan (SUNMEDICA Co., Japan) is widely used in dentistry as a bonding agent.[6] Adhesive resins have been used to seal dentine, much like enamel, through the creation of hybridized dentine that protects the pulp from the actions of oral fluids and their contaminants. Recently, a 4-META resin sealer containing radiopaque zirconium oxide has been developed as a root canal sealer.[7] This modified type of 4-META resin adheres to root canal dentine and it should be expected to seal the root canal

system more effectively through the formation of a resin impregnation layer.

There have been several studies concerning the cytotoxicity and tissue responses after 4-META resin application *in vitro* and *in vivo*.[8-12] The 4-META resin penetrated into the pulp tissue and polymerized to form a connective tissue-resin layer. This layer is thought to elicit macrophage migration subjacent to the interface of the 4-META resin-dental pulp tissue.[13] However, little is known about how 4-META resin controls the mechanism of intracellular molecular events as signal transduction. The present study examined the gene expression patterns using a human genome expression microarray and real-time PCR analyses in hard tissue forming osteoblasts cultured with a 4-META resin sealer under the clinically simulated condition.

MATERIALS AND METHODS

Cell proliferation study

To identify the polymerizing effect and to examine the cytotoxicity of the cylindrical block of 4-META resin sealer (5 mm in diameter, 10 mm in length), the resin block was placed on a 100-mm culture dish. Osteoblasts (NOS-1 cells[14]) derived from human osteosarcoma were seeded at a density of 4×10^6 cells in α-MEM containing 10% FBS and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and air. The standard power-liquid ratio (sealer powder: 0.37g, monomer: 4 drops, catalyst: 1 drop) according to the manufacturer's instruction was used in this study. This shape and size was stable in cell culture system and suitable for cell

growth and proliferation. After 3 days of culture, the number of cells was counted using a hemocytometer under a phase-contrast microscope. Values were expressed as mean ± SE, and the difference between the control and 4-META resin sealer groups was compared using Student's *t* test. A *p* value of less than 0.05 was considered significant.

Isolation of RNA

NOS-1 cells were seeded in a 100-mm culture dish at a density of 4 x 10^6 cells. After 3 days of culture with or without a 4-META resin sealer, the cells were rinsed twice with phosphate-buffered saline (-) and then were scraped off the dishes with a rubber policeman. Total RNA was prepared using an Atlas™ Glass Total RNA Isolation Kit (Takara-Bio.Co., Japan), according to the instructions provided by the manufacturer.

Microarray hybridization and signal analysis

A cDNA probe was synthesized from total RNA isolated as described above. An in vitro transcription reaction was then done to produce biotin-labeled cRNA from the cDNA. The cRNA was fragmented before hybridization. A hybridization cocktail was prepared, including the fragmented target, probe array controls, BSA, and herring sperm DNA. It was then hybridized to the probe array during a 16-hr incubation. Streptavidin Phycoerythrin was used to stain the probe array. The data were analyzed using the GeneChip® Operating Software program (Affymetrix, MA, USA).

Real-time PCR

NOS-1 cells were similarly seeded in a 100-mm culture dish at a density of 4 x 10^6 cells and cultured for 3 days in α-MEM containing 10% FBS with or without 4-META resin. Total RNA was extracted using Trizol® reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA synthesis and amplification via PCR were performed using the SuperScript™ First-Strand Synthesis System (Invitrogen) and an oligo dT primer. cDNA obtained from 400 ng of total RNA was used for one PCR. For real-time PCR reaction, a master mix of the following reaction components was prepared to the indicated quantities: 9.1 μl of water, 0.3 μl of diluted reference dye, 0.2 μl of forward and reverse primers (details in Tables I) and 10 μl of Brilliant® SYBR® Green QPCR master mix (Stratagene, CA, USA). The above-mentioned reaction was placed in a 0.2 ml tube and 0.2 μl of cDNA was added as the PCR template. The PCR sequence protocol was as follows: denaturation program (94 ^oC, 1 min), amplification and quantification program (56 ^oC, 1 min) and extension program (72 ^oC, 1 min) repeated 40 times. The amplification and data acquisition were carried out using an Mx3000P QPCR machine (Stratagene).

Quantification was accomplished by determining the threshold cycle (the second derivative of the resulting fluorescence curve) at which the amplicon is detected during the PCR and then comparing this to the standard curve calculated from the parallel quantification reactions. These calculations were done with the comparative quantification method using the Stratagene

form. These data were normalized by glyceraldehyde phosphate dehydrogenase (GAPDH).

RESULTS

The number of cells in the control group was (8.21 $\pm$ 0.17) x 10^6 (n = 3), and that in the 4-META resin adhesive sealer group was (7.94 $\pm$ 0.09) x 10^6 (n = 3) after 3 days of incubation. There was no significant difference between the control and adhesive sealer groups.

The signal levels of individual cRNA from the experimental and control groups were compared after hybridization on a human genome expression microarray which consisted of about 54,000 probe sets. The data images were analyzed for probe intensities; and the results were reported in tabular and graphical formats. Using such analyses, 6 clones concerning coding for various cell proliferation and/or signaling-related molecules exhibited a balanced difference score between the experimental and control groups of $\geq$2.0. Table II shows the expressions of various genes in the 4-META resin sealer group in comparison to the control group.

A real-time PCR analysis revealed that mRNA coding for 4 signaling-related genes, integrin β, TGF β−stimulated protein, craniofacial development protein 1, and phosphoinositide-3-kinase (PI3K), was clearly increased after 3 days of culture with a 4-META resin sealer with a balanced score ratio between both groups of $\geq$5.0 (Table Ⅱ). The integrin β and PI3K genes showed extremely high ratios.

DISCUSSION

This study was carried out to demonstrate the effects of components dissolved from 4-META resin sealer on osteoblasts in culture. The relationship between increased gene expression and intracellular signal transduction molecules was investigated using a human genome expression microarray; real-time PCR, and an antibody assay for phosphorylation of MAPKs. Although there was no significant difference in the cell number between the control and adhesive sealer groups, a high expression of integrin, TGF-β related protein, craniofacial development protein 1, and PI3K genes was clearly demonstrated in the experimental group.

Integrins are cell surface receptors that in their active form are composed of two subunits: α and β.[15,16] They are the major structural receptors that maintain proper tissue organization through cell-cell and cell-extracellular matrix interactions. Integrins also participate in the transferring of cellular signals, thus leading to the modulation of cell death or survival, proliferation and migration.[17,18] Cell signaling induced by integrins is also associated with cytoskeleton reorganization following cell spreading and migration,[19,20] as well as the modulation of cell proliferation. α9β1 integrin is a signaling receptor for nerve growth factor, which activates the MAPK (ERK1/2) pathway. Thyroid hormone also stimulates ERK activation in the human osteoblast-like cell line via integrin $\alpha_V\beta_3$ and one functional effect of this ERK activation is increased DNA synthesis.[21]

The TGF-β family includes many related factors that have diverse functions during embryonic development and adult tissue homeostasis.[22-25]

TGF-β and related factors use a simple mechanism to signal to the nucleus. They bind to membrane receptors that have a cytoplasmic serine/threonine kinase domain. Binding of the ligand causes the assembly of a receptor complex that phosphorylates the SMAD family related proteins that bind DNA and recruit transcriptional co-activators or corepressors.[22] TGF-β1 enhances the proliferative, migratory, and adherent abilities of adventitial fibroblasts together with the upregulation of the expression of cytokines in cross-talk among the SMAD, MAPK, and integrin signaling pathways.[26]

Craniofacial development protein 1 gene is associated with anti-apoptosis, cell adhesion, multicellular organization development, regulation of cell proliferation, and regulation of cell shape.[27]

PI3Ks, which represent a family of lipid kinases, are key mediators of intracellular signaling in many cell types.[28] PI3Ks are upstream regulators in a number of signaling cascades that control proliferation, growth, cell death, migration, metabolism, and a host of other biological responses.[29] Furthermore, the PI3K/Akt pathway is one of the most critical signaling pathways involved in the regulation of cell survival.[30,31] For example, TGF-β2-induced epithelial-mesenchymal transition in postoperative remnants of lens epithelial cells is mediated by the downregulation of connexin 43, which is regulated through the PI3K/Akt pathway.[32]

There have been several reports addressing the histopathological findings and reactions in cell culture[33] after the application of 4-META resin. Nerve regeneration and proliferative activity in amputated dog pulp tissue were investigated after the application of 4-META resin.[9] Although cell

differentiation and nerve regeneration are delayed in the experimental group, wound healing was the same as in the calcium hydroxide control group. Rabbit pulp tissue was immersed in MMA or in 5% 4-META/MMA and autotransplanted beneath a kidney capsule. MMA does not inhibit the osteogenic activity of pulp tissue, while 5% 4-META/MMA inhibits osteogenic activity to some extent.[10] In *in vitro* cell culture studies, MMA showed little cytotoxicity and small changes in dipalmitoylphosphatidylcholine liposomes, whereas *n*-butytyl acrylate showed increased cytotoxicity and large changes in the liposomes with membrane disturbance.[8] In an MTT assay of human pulp cell viability, the cytotoxicity of 4-META/MMA-TBB was comparable with that of MMA-TBB. TBB showed higher cytotoxicity than 4-META/MMA. The cytotoxicity induction of a 4-META resin may be preferably associated with TBB.[11] In culture studies using a rat dental pulp cell line, polymerized 4-META resin did not induce a cytotoxic response even at 14 days.[12] The main and first findings were that the integrin β and PI3K genes showed extremely high ratios. These genes are relating to cell adhesive and proliferation and differentiation. Although further studies are necessary to identify the specific components of 4-META resin adhesive sealer that affect cell viability, these data together with the present findings suggest that MMA released from 4-META resin sealer is a probable candidate for activating such intracellular events as signal transduction.

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Table Ⅰ. Oligonucleotide primers used for PCR analyses of genes in relation to cell proliferation and signaling

Gene name		Oligonucleotides
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Forward	GACAAGGGCTCTGGAGACAG
	Reverse	ACTGGTGAGCTTTCGCATCT
Transforming growth factor beta-stimulated protein TSC-22	Forward	GCTTGTAGTGGCATGAGGTG
	Reverse	TTTGTGTGGCCTGTGAGATT
GTPase, IMAP family member 1	Forward	GCTCATCCTTGTTGGGAGAA
	Reverse	GCTGAAAATGTCCGGAGTGT
Craniofacial development protein 1	Forward	CTGCGGTCTTGTGAGTTTGA
	Reverse	TCCCTTGGGTTTTCTGTGTC
Calmodulin-like 3	Forward	CATCTGAATGACACGGAACG
	Reverse	GATCTTGCCTGGTGCCTAAG
Phosphoinositide-3-kinase, class 2, alpha polypeptide	Forward	GTTGTCAAGCAGCACCAGAA
	Reverse	CTCGAAACTGTCATCCAGCA
GAPDH	Forward	CCATGGAGAAGGCTGGGG
	Reverse	CAAAGTTGTCATGGATGACC

Table Ⅱ. Representative gene expression after 3 days of culture corresponding to microarray experiment

Gene name	Ratio	Confirmation[a]
Response to abiotic stimulus		
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	5.3	409
Cell-cell signaling		
Transforming growth factor beta-stimulated protein TSC-22	2.6	5.0
Signal transduction		
GTPase, IMAP family member 1	2.6	1.3
Cell growth		
Craniofacial development protein 1	2.5	4.7
Calcium ion binding		
Calmodulin-like 3	3.0	1.0
Kinase		
Phosphoinositide-3-kinase, class 2, alpha polypeptide	2.1	13.2

[a]Confirmed by quantitative PCR data (data from triplicate samples).