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Tannin-fluoride preparation attenuates prostaglandin E_2 production by dental pulp cells

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Abstract. Glass ionomer cements (GICs) are widely used for the operative restoration of dental caries. However, it has been reported that the components of GICs cause pulpal inflammatory responses. Recently, GICs containing tannin-fluoride preparation (HY agent) were developed. In this study, we investigated the effect of HY agent on prostaglandin E_2 (PGE_2) release from GIC-stimulated rat dental pulp cells (RPC-C2A). Extracts derived from GIC disks were used with HY(+) and without HY(-) agent. After treatment with GIC extracts, ATP contents, COX-2 mRNA and protein expression in RPC-C2A cells, and PGE_2 production in culture media were analyzed. HY agent suppressed HY(-)-stimulated PGE_2 release from RPC-C2A cells, as well as COX-2 mRNA and protein expression. Moreover, tannic acid attenuated COX-2 mRNA induced by HY(-) extract in a dose-dependent manner. Taken together, these results suggest that tannic acid in HY agent may suppress GIC-induced production of PGE_2 by inhibition of COX-2 expression in dental pulp cells.

Introduction

Dental pulp cells are damaged by various factors, including caries, attrition, bruxism and restorations, and are stimulated to form reactionary dentin (1). The dental pulp changes after dentinal injury and cavity preparation are caused by displacement of the odontoblast layer (2). There are 45,000/mm^2 dentinal tubules near the pulp (3). Therefore, the components of restorative materials have a significant effect on the pulp. Furthermore, it has been reported that marginal leakage and subsequent extract of the restorative material induces pulp inflammation (4).

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Glass ionomer cements (GICs) have a good biocompatibility and are widely used for the operative restoration of dental caries. However, GICs show cytotoxicity when in direct contact with the dental pulp, and GIC fillings induce pulp inflammation in more than 20% of the teeth after 30 days (5).

To solve these problems, GICs, including tannin-fluoride preparation (HY agent), have been developed. Upon addition of HY agent, the amount of fluoride release increases and fluoride penetrates into deep regions of the dentin faster. Crystal growth occurs, thus closing the dentinal tubules (6). The decreased stimulation by sclerosis of dentinal tubules helps to preserve the dental pulp. Filling the gap between dentin and material by crystal growth is effective for the prevention of microleakage.

Tannic acid, the main component of HY agent, suppresses inflammation and joint damage in rheumatoid arthritis (7). Furthermore, tannic acid potently inhibits phorbol ester-induced nitric oxide generation in rat hepatocytes (8), and it is known that tannic acid shows anti-inflammatory effects owing to its astringent properties (9).

Nevertheless, the effects of HY agent and tannic acid on dental pulp remain unknown. The objective of this study was to evaluate the effect of HY agent on the production of prostaglandin E_2 (PGE_2) by dental pulp cells.

Materials and methods

Restorative materials and cement extract. Glass ionomer F (Shofu, Kyoto, Japan) was used as the glass ionomer cement containing HY agent HY(+) in this study. Glass ionomer F without HY(-) agent was also used. The materials were fabricated in sterile teflon molds 6 mm in diameter and 3-mm thick. The materials were packed into the mold and allowed to set for 24 h after mixing. The specimens were immersed in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) at 4˚C for 7 days (cement extract).

Cell culture. The rat clonal dental pulp cell line RPC-C2A (10) was used in the present study. RPC-C2A cells were grown in cement elution supplemented with 10% fetal bovine serum (FBS) and 66.7 μg/ml kanamycin-sulfate at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. DMEM was employed as a control.

ATP contents in RPC-C2A cells. To measure the cell ATP content, the ViaLight Plus® Cell Proliferation and Cytotoxicity BioAssay kit (Lonza, Rockland, ME, USA) was used. The cells were cultured with cement extract for 24 h. Light emission levels were measured using a Wallac 1420 ARVOsx multi level counter (PerkinElmer, Boston, MA, USA).

Enzyme immunoassay. PGE_2 levels were measured with the Amersham Prostaglandin E_2 Biotrak Enzyme immunoassay system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of COX-2 mRNA. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The RNA (1 μg) was denatured and used to synthesize cDNA with ReverTra Ace-α-™ (Toyobouseki, Osaka, Japan) following the manufacturer's instructions. RT-PCR amplification was performed with Ampli *Taq* Gold® polymerase (Applied Biosystems, Foster City, CA, USA) and specific primers designed for rat COX-2 (5'-CCGGGTTGCTGGGGG AAGGA-3' and 5'-CCACCAGCAGGGCGGGATACAG-3'), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (5'-CGGAGTCAACGGATTTGGTCGTAT-3'and5'-AGCCTT CTCCATGGTGGTGAAGAC-3') and L19 (5'-CTGAAG GTGAAGGGGAATGTG-3' and 5'-GGATAAAGTCTTGAT GATCTC-3'). Amplification was performed at 30 cycles for COX-2, 23 cycles for GAPDH and 38 cycles for L19. Each cycle consisted of 1 min of denaturation at 94˚C, 1 min of annealing at a specific temperature for each set of primers, and 1 min of extension at 72˚C (10 min in the last cycle; Applied Biosystems). The PCR products were electrophoresed on 2% agarose gel. The levels of mRNA expression were analyzed with Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA, USA) and normalized with GAPDH mRNA.

Western blot analysis. After treatment, confluent cell monolayers in 100-mm dishes were washed by PBS. Then, the cell lysates were collected using lysis buffer [10 mM HEPES-KOH (pH 7.5), 100 mM KCl and 0.1% NP-40]. Aliquots containing 15 μg total protein were separated by reducing 10% polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked for 1 h at room temperature in Immuno Block (DS Pharma Biomedical, Osaka, Japan). The membranes were thoroughly washed with TBS-T [40 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.3% Tween-20] and incubated in the presence of COX-2 antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Then, the membrane was washed with TBS-T and incubated in horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. For the detection of actin, HRP-conjugated goat polyclonal antibodies against actin (Santa Cruz Biotechnology) were used. Immunoreactive proteins were detected with the enhanced chemiluminescence assay Western blotting detection system (PerkinElmer).

Statistical analysis. The results were statistically analyzed by the Tukey test for multiple comparisons after one-way analysis of variance (ANOVA). The level of significance was set at $P<0.05$ or 0.01.

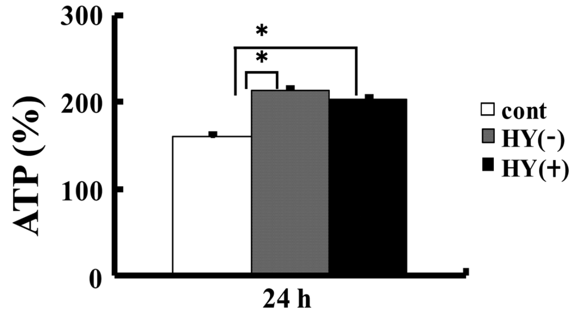

Figure 1. ATP contents in RPC-C2A cells. RPC-C2A cells were cultured with cement extract for 24 h and ATP contents were measured. The value before culture by cement extract was 100%. Data are the means ± SD (n=12). *P<0.05. cont, DMEM; HY(-), cement extract without HY agent; HY(+), cement extract with HY agent.

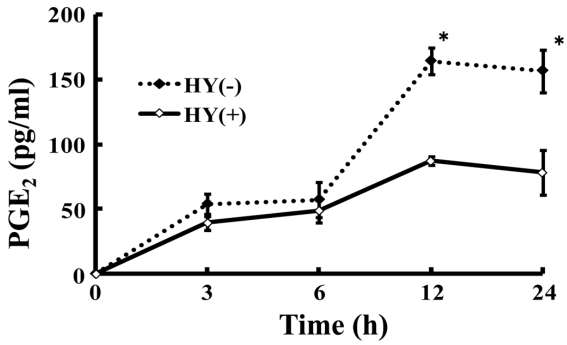

Figure 2. HY(+) extract reduced GIC-stimulated PGE_2 production. Conditioned medium derived from RPC-C2A cells stimulated by HY(-) and HY(+) for the indicated times was analyzed for PGE_2 by ELISA. Data are the means ±SD (n=3). *P<0.01 vs. HY(+) values at the same point. HY(-), cement extract without HY agent; HY(+), cement extract with HY agent.

Results

Effect of HY agent on ATP contents in GIC-stimulated RPC-C2A cells. It has been reported that original extracts of GICs have cytotoxicity (11). To examine whether HY agent increases the cytotoxicity of GIC-treated cells, RPC-C2A cells were stimulated with HY(-) or HY(+) extract, and ATP contents were measured in those cells (Fig. 1). ATP contents were significantly increased after 24 h of stimulation with HY(+) and HY(-) extract (Fig. 1).

GICs induced PGE_2 release from RPC-C2A cells. To address the effect of HY agent on the secretion of PGE_2 by GIC-stimulated RPC-C2A cells, the amount of PGE_2 protein was determined by ELISA using a specific antibody against murine PGE_2. As shown in Fig. 2, the results of ELISA revealed that the amount of PGE_2 in the culture medium was markedly increased 12-24 h after GIC treatment, and that HY agent significantly suppressed this accumulation.

HY agent down-regulated GIC-stimulated COX-2 mRNA and protein expression in RPC-C2A cells. The conversion of arachidonic acid to PGH_2 by COX is one of the main metabolic

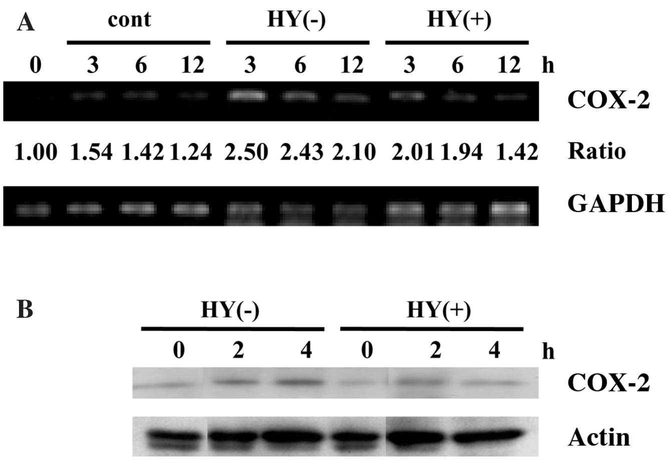

Figure 3. Induction of COX-2 expression by cement extract in RPC-C2A cells. RPC-C2A cells were treated with cement extract for the indicated times. (A) Total RNA from RPC-C2A cells was analyzed by RT-PCR to measure COX-2 or GAPDH mRNA levels as a control. (B) Western blot analysis of COX-2 protein levels from total extracts of RPC-C2A cells treated with cement extract. Cont, DMEM; HY(-), cement extract without HY agent; HY(+), cement extract with HY agent.

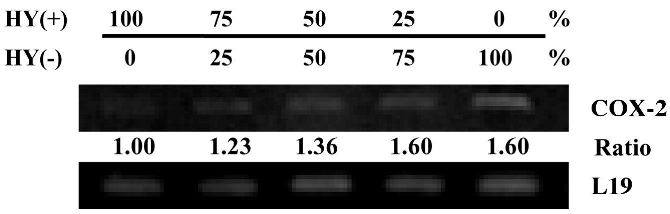

Figure 4. Effect of HY agent on GIC-induced COX-2 mRNA expression. RPC-C2A cells were cultured and then extract derived from HY(-) or HY(+) was added at the final ratio indicated. COX-2 mRNA levels were normalized to the expression of L19 mRNA and a ratio against HY(+). HY(-), cement extract without HY agent; HY(+), cement extract with HY agent.

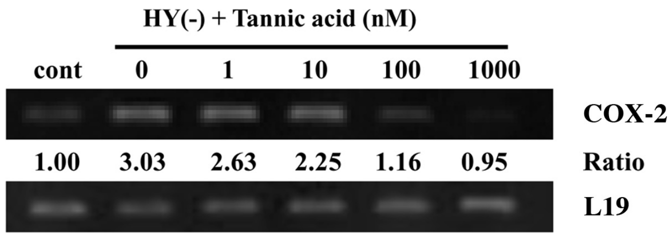

Figure 5. Tannic acid inhibits GIC-stimulated COX-2 mRNA expression. Representative RT-PCR images for COX-2 mRNA obtained from RPC-C2A cells stimulated by cement extract. RPC-C2A cells were cultured in HY(-) medium with tannic acid at the indicated concentrations for 3 h. COX-2 mRNA levels were normalized to the expression of L19 mRNA, and a ratio to the control is shown. Cont, DMEM; HY(-), cement extract without HY agent.

steps of PGE_2 synthesis (12). It was shown that the expression of COX-2 is induced by various inflammatory conditions. However, it remains to be clarified whether HY agent inhibits this procedure. In order to address these issues, the expression of COX-2 was examined in dental pulp cells. As shown in Fig. 3A, feeble expression of COX-2 mRNA was constitutively observed in RPC-C2A cells. An increase in COX-2 mRNA was apparent as early as 3 h after the addition of HY(-) extract, and then decreased gradually. By contrast, the addition of HY agent HY(+) partially blocked GIC-induced COX-2 mRNA. Likewise, whether HY agent actually inhibits the production of COX-2 protein by GIC-stimulated dental pulp cells was investigated; the amount of COX-2 protein was determined by Western blot analysis using a specific antibody against murine COX-2. These results were also confirmed using dilution experiments of GIC extracts. In agreement with the mRNA expression detected, the results of Western blot analysis revealed that the amount of GIC-induced COX-2 was clearly decreased (Fig. 3B). As expected, the suppressive effect of HY agent on COX-2 mRNA was dose-dependent (Fig. 4). A clean-cut decrease in COX-2 mRNA was observed at 100% HY(+).

Tannic acid is the main component of HY agent. The above findings led us to explore the possibility that tannic acid is capable of abolishing COX-2 mRNA expression in RPC-C2A cells treated by GICs without HY agent. Tannic acid significantly reversed the COX-2-inducible effect of HY(-) extract (Fig. 5).

Discussion

One of the purposes of operative dentistry is to maintain pulpal health in a compromised tooth. The materials used for operative dentistry should not cause toxicity or injury to the dental pulp. GICs are well known to be biocompatible and have been widely used for direct pulp capping. By contrast, inflammation of the dental pulp without bacteria has been shown 30 days after filling cavities with GICs (5). GICs are composed of approximately 10% acrylic acid. It was reported that acrylic acid implanted subcutaneously in rats led to a chronic inflammatory reaction with macrophage infiltration (13). This led us to address the cytotoxicity of GIC extracts. To examine whether GIC extracts suppress the cell proliferation of dental pulp cells, the level of ATP contents was measured at 24 h. Unexpectedly, ATP contents in GIC-stimulated RPC-C2A cells increased as compared to the control (Fig. 1). Stimulation of GICs did not induce significant cytotoxicity.

GICs exhibit the ability of fluoride release and pulp biocompatibility. However, conflicting results have been reported concerning the effects of GICs on various cells. The cell proliferation of mouse fibroblasts was increased by GIC extract (14); this finding is consistent with our results. On the other hand, Lewis *et al* reported that the numbers of hamster cheek pouch cells were decreased by GIC extract (15). These discrepancies can be attributed to several factors, including cell specificity, ratio of material and medium, incubation temperature and exposure time.

PGE_2 is a key chemical mediator generated from arachidonic acid (16). Therefore, the effects of GIC extract on PGE_2 production by RPC-C2A cells were also examined. HY(-) extract induced PGE_2 production in RPC-C2A cells, but adding HY agent suppressed this PGE_2 production (Fig. 2). Interleukin-1β (IL-1β) and TNF-α stimulate the PGE_2 production of mouse osteoblasts (17). In addition, BisGMA, resin monomer released from composite resin, induces PGE_2 production in dental pulp cells (18). Likewise, we demonstrated that the GIC extract stimulated PGE_2 production.

COX-2 is an important enzyme in the pathway, by which arachidonic acid is converted to prostaglandins (19-21).

Therefore, we focused on COX-2 production induced by GICs in dental pulp cells. The GIC extract induced COX-2 mRNA expression in RPC-C2A cells (Fig. 3A). Dentin bonding agents were shown to induce COX-2 mRNA expression in human pulp cells (22). Moreover, resin monomer, triethyleneglycol dimethacrylate (TEGDMA) and 2-hydroxyethyl methacrylate (HEMA) were found to induce COX-2 mRNA expression (23). By contrast, the COX-2 mRNA and protein expression induced by HY(-) extract was decreased by HY agent (Fig. 3A and B). In addition, HY agent reduced HY(-)-induced COX-2 mRNA expression in RPC-C2A cells in a dose-dependent manner (Fig. 4). Furthermore, tannic acid, the main component of HY agent, also reduced COX-2 mRNA induction in HY(-)-stimulated RPC-C2A cells (Fig. 5). Tannic acid exerted a potent anti-inflammatory effect by activation of the transcription factors (24). It was reported that 100 μM tannic acid increased COX-2 and iNOS expression in murine macrophages (25). We found that 1 μM tannic acid suppressed COX-2 mRNA expression, whereas 10 μM tannic acid induced COX-2 mRNA expression (data not shown). Taken together, tannic acid may have contradictory effects on COX-2 mRNA expression depending on its concentration. The distinction between these properties of tannic acid warrants further investigation.

In conclusion, the present study demonstrated that HY agent suppressed GIC-induced PGE_2 production via the inhibition of COX-2 expression.

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