Original Article

Orthodontic Adhesives Induce Human Gingival Fibroblast Toxicity and Inflammation

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ABSTRACT

Objective: To test the null hypothesis that the resin base and the resin hybrid glass ionomer base adhesives do not cause inflammation after contacting primary human gingival fibroblasts in vitro.

Materials and Methods: The resin base and resin hybrid glass ionomer base adhesives were used to treat human gingival fibroblasts to evaluate the survival rate using MTT colorimetric assay to detect the level of cyclooxygenase-2 (COX-2) mRNA by reverse transcription polymerase chain reaction (RT-PCR) technique and COX-2 protein expression using Western blot analysis. The results were analyzed using one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed using the Tukey test and a value of $P < .05$ was considered statistically significant.

Results: The paste and primer of the resin base adhesive and the liquid of glass ionomer adhesive showed decreasing survival rates after 24 hours of treatment ($P < .05$). All orthodontic adhesives induced COX-2 protein expression in human gingival fibroblasts. The exposure of quiescent human gingival fibroblasts to adhesives resulted in the induction of COX-2 mRNA expression. The investigations of the time-dependent COX-2 mRNA expression in adhesive-treated human gingival fibroblasts revealed different patterns.

Conclusions: The hypothesis is rejected. For orthodontic patients with gingival inflammation, except for those with oral hygiene problems, the activation of COX-2 expression by orthodontic adhesive may be one of the potential mechanisms.

KEY WORDS: Orthodontic adhesive; Inflammation; COX-2; Survival rate

INTRODUCTION

An ideal orthodontic adhesive should have good chemical and physical properties, be nonirritating to surrounding tissue and be easy to manage. Orthodontic adhesives are used to glue attachments directly to the surface of the tooth. There are two types of adhesive material applied in orthodontic attachment bonding. They are resin base and resin hybrid glass ionomer base adhesives. Some of the adhesives directly make contact with soft oral tissue and cause tissue irritation.

Ruyter and Svebdseb[1] reported that resin-based filling materials and sealants may contain up to 50% remaining, unreacted component groups. Ideally, the agents should be placed in the oral cavity without coming into contact with the gingiva or other oral mucosa, but unintentional spillage of adhesive to the soft tissue may occur sometimes. In an animal study, adhesives may cause inflammatory reactions in the mucosa of dogs.[2]

Both resin base and resin hybrid glass ionomer base adhesives can cause cytotoxicity.[3–6] In our previous study, we showed that the degree of adhesive toxicity was different according to different cell culture origins.[7]

The prostaglandin E2 inflammatory mediator is known to exert diverse physiologic actions in different tissues and to be involved in the inflammation process.[8–10] The enzymes, including phospholipase A2 and cyclooxygenase (COX), regulate the production of

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Table 1. Nucleotide Sequence and Size of the Expected PCR Products for Oligonucleotide Primers Used for RT-PCR[a]

Gene	Sequence	PCR Product, bp
COX-2	Forward: 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′	232
	Complement: 5′-AGCAATTTTCCCACAATCTCATTTGAA-3′	
	Reverse: 5′-AGATCATCTCTGCCTGAGTATCTTT-3′	
	Complement: 5′-AAAGATACTCAGGCAGAGATGATCT-3′	
GAPDH	Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′	207
	Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′	

[a] PCR indicates polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair.

the prostaglandin. Prostaglandins are produced by the action of COX enzymes on the free arachidonic acid liberated from membrane phospholipids by phospholipases. Prostaglandin endoperoxide H synthase (also referred to as COX) is the rate-limiting enzyme for the production of prostaglandins and thromboxanes from free arachidonic acid.[11] Two forms of COX have now been described: a constitutive enzyme (COX-1), present in most cells and tissues,[12] and an inducible isoenzyme (COX-2) expressed in response to cytokine growth factor, lipopolysacharride, and other stimuli.[13,14]

COX-2 is an intermediate response gene that encodes a *Mr* 71000 cytoplasmic protein that is up-regulated at sites of inflammation.[15] COX-2 is constitutively expressed in the brain, kidney, and testes; however, in most other tissues its expression is induced by proinflammatory or mitogenic agents, including cytokines, tumor promoters, endotoxins, and mitogens.[16] So far there has been little information written about the inflammation reactions after adhesives contact human cells.

In orthodontic treatments, controlling periodontal tissue health is important. It is hypothesized that the orthodontic adhesives can induce gingival inflammation. The purpose of the present study was to evaluate in vitro inflammation behavior of the resin base and resin modified glass ionomer base adhesives after contacting primary human gingival fibroblasts.

MATERIALS AND METHODS

Material Preparation

The adhesive used in the present study was the resin base adhesive, Transbond XT (Unitek, 3M, Monrovia, Calif), and resin hybrid glass ionomer adhesive, Multi-Cure Glass Ionomer Cement (Unitek, 3M) (Table 1). The adhesives were mixed or cured according to the manufacture's instructions. The experiment groups were divided into the Transbond XT paste (paste), Transbond XT primer (primer), Transbond XT curing (XT-mixed), Multi-Cure Glass Ionomer Cement powder (powder), Multi-Cure Glass Ionomer Cement liquid (liquid), and Multi-Cure Glass Ionomer Cement mix (GI mixed) groups. The above materials weighed 4 g and were immersed in 10 mL dimethyl sulfoxide (DMSO) solution for 7 days at 37°C in an incubator.

Human Primary Gingival Fibroblast Culture

Gingival tissues were obtained by excision of bicuspid gingiva from a 12-year-old female patient who was undergoing orthodontic treatment. Parents signed the informed consent before donating the gingival specimen. The resultant tissues were cut into 1–2 mm^3 pieces, washed twice with phosphate-buffered saline (PBS) supplemented with 100 U/mL penicillin (Sigma Chemical, St Louis, Mo), 100 μg/mL streptomycin (Sigma), and placed into 25 cm^3 tissue-culture flasks.

The explants were incubated with culture medium consisting of alpha minimum essential medium (α-MEM; Sigma), 30% fetal bovine serum (FBS, Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, at 37°C in a humidified atmosphere of 5% carbon dioxide in air. When the outgrowth cells were observed in the cultures, the medium was replaced twice sequentially, and the cells were reincubated until the proliferating cells had reached confluence. The cells were detached from the monolayer using brief treatment with 0.02% trypsin/0.04 M EDTA and recultured in 100 cm^2 tissue culture flasks until confluent monolayers were again obtained. Cells between the fifth and seventh passages were used for the experiments described below.

Cell Viability Test—MTT Colorimetric Assay

The MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay measure of succinic dehydrogenase activity giving an indication of the level of cell respiration was adopted for this experiment.

Human gingival fibroblasts were inoculated into 96-well plates (Falcon, NJ) at a density of 4×10^3 cells/well, at 37°C, placed in a 5% CO_2 incubator for 2 days. After incubation, cells were treated with 10 μL of various experiment solutions of paste, primer, XT-mixed, powder, liquid, and GI mixed for 1, 3, 6, 12, and 24 hours; 50 μL of MTT dye was then added to each well. Plates were incubated in a CO_2 incubator for 4 hours. After formazan formed, it was dissolved in isopropa-

nol. For each well, the degree of light absorbance at 570 nm was then measured with an enzyme-linked immunosorbent assay reader (U2000, Hitachi, Tokyo, Japan). The cell viability results were presented as the ratio (%) of the absorbance at 570 nm in the experimental wells to that detected in the control wells. Five replicates of each concentration were performed for each test. All assays were repeated three times to ensure reproducibility.

Western Blot Analysis—COX-2 Protein Evaluation

A Western blot is a method to detect a specific protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide or by the 3-D structure of the protein (native/nondenaturing conditions). The proteins are then transferred to a membrane, where they are probed using antibodies specific to the target protein. The present study applied this method to detect the COX-2 protein expression.

The Western blot assay was performed as described in our previous study.[17] The cells were solubilized with SDS buffer (1 mM $MgCl_2$, 50 mM Tris-HCl, 5 mM EDTA, pH 7.5 and 0.5% Trition X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 minutes on ice. Then, cell lysates were centrifuged at 12,000 $\times$ *g* at 4°C, and the protein concentrations were determined with Bradford reagent with the use of bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% BSA for 2 hours, rinsed, and then incubated with primary antibodies anti-COX-2 diluted 1:1000 in PBS containing 0.05% Tween 20 for 2 hours. After three washes with Tween 20 for 10 minutes, the membranes were incubated for 1 hour with biotinylated secondary antibody (polyclonal anti-rabbit IgG for COX-2) diluted 1:2000 in the same buffer, washed again as described above and treated with 1:2000 streptavidin-peroxidase solution for 30 minutes. Beta-actin antibody was used in Western blot as a control. After a series of washing steps, the reactions were developed with the use of diaminobenzidine. The intensities of the obtained bands were determined with the use of a densitometer (AlphaImager 2000).

Reverse Transcription Polymerase Chain Reaction Analysis—COX-2 mRNA Expression

In molecular biology, reverse transcription polymerase chain reaction (RT-PCR) is a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. The exponential amplification via RT-PCR provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected. Thus, by applying this method one can detect the COX-2 mRNA expression.

Following the product's instruction, total RNA was prepared with the use of a TRIzol reagent (Gibco, Grand Island, NY). Single-stranded DNA was synthesized from RNA in a 15-μL reaction mixture containing 100 mg random hexamer and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco). The reaction mixture was diluted with 20 μL of water, and 3 μL of the diluted reaction mixture was used for polymerase chain forward and reverse primers and 2 U of Taq DNA polymerase. Amplification was performed at 25 cycles for glyseraldehyde-3-phosphate dehydrogenase (GAPDH) and 30 cycles for COX-2 in a thermal cycle. Each cycle consisted of 1 minute of denaturation at 94°C, 1 minute of annealing at 57°C, and 1 minute of extension at 72°C. The sequences of primers employed are listed in Table 1. The PCR products were analyzed using agarose gel electrophoresis. The intensity of each band, after normalization with GAPDH mRNA, was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, Calif).

Statistical analysis was conducted using the program SAS for Unix 6.09 (SAS Institute, Cary, NC) with one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed using the Tukey test, and a value of $P < .05$ was considered statistically significant.

RESULTS

Survival Rate

The survival rate of primary gingival fibroblasts showed that the resin hybrid glass ionomer liquid group had lower survival rates after 6, 12, and 24 hours of treatment ($P < .05$) compared with the other groups. The powder and mixed resin hybrid glass ionomer adhesive groups showed no differences after the different treatment times ($P > .05$) (Figure 1).

The survival rate of the Transbond XT resin paste group and the primer group showed decreasing survival rates after 24 hours of treatment ($P < .05$). The mixed Transbond XT resin adhesive group showed no statistical differences at the different times of treatment ($P > .05$) (Figure 1).

COX-2 Protein Expression

In Western blot analysis, results showed that the expression of COX-2 protein in human gingival fibro-

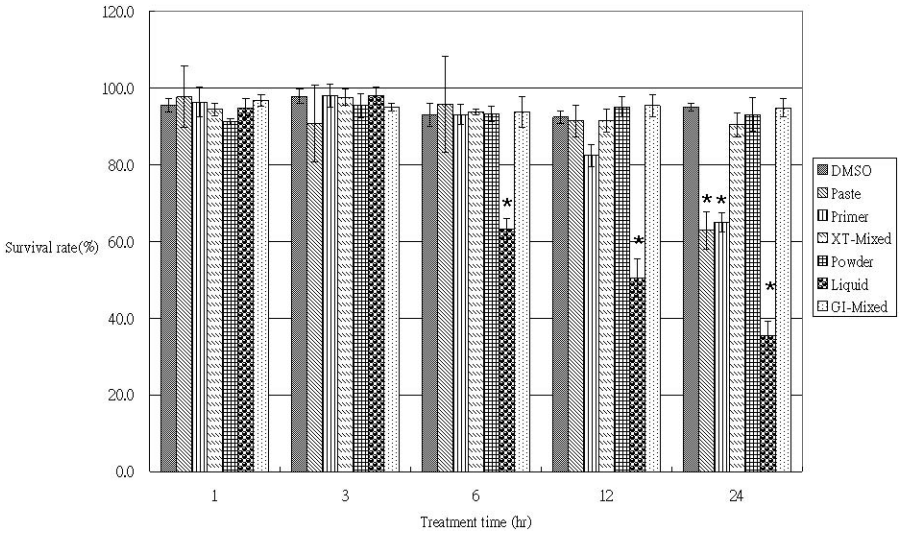

Figure 1. The survival rate (%) of primary gingival fibroblasts treated with 10 μL of various solutions of paste, primer, XT-mixed, powder, liquid, and GI mixed for 1, 3, 6, 12, and 24 hours. The liquid of resin hybrid glass ionomer group showed lower survival rate of gingival fibroblasts at 6, 12, and 24 hours (*$P < .05$). The paste and primer of the resin base adhesive group and the liquid of the glass ionomer adhesive group showed decreasing survival rates at 24 hours of treatment.

blasts was seen in all experimental groups (Figure 2). The ratio of the COX-2 protein expression from the densitometer measurements showed statistical significance was evident for the primer of the resin base group after 6 hours of treatment. The COX-2 protein ratios of the primer group from the densitometer were detected at 1, 3, 6, 12, and 24 hours with values as follows: 1.16, 1.15, 1.09, 1.27, and 2.17 ($P = .013, < .05$). The paste ($P = .98$) and mixed ($P = .94$) groups of the resin base adhesive both showed no statistical differences in the ratios ($P > .05$) (Figure 2).

The COX-2 protein expression of the powder of resin hybrid glass ionomer group showed an increase after 12 hours of treatment ($P < .05$). The densitometer ratio at 1, 3, 6, 12, and 24 hours had the values as follows: 0.75, 0.77, 0.56, 0.74, and 1.43. The liquid of the resin hybrid glass ionomer adhesive group showed the highest COX-2 protein expression (2.77) after 24 hours of treatment. However, the mixed resin hybrid glass ionomer adhesive group showed no statistical differences on the COX-2 protein expression at the different treatment times ($P < .05$) (Figure 2).

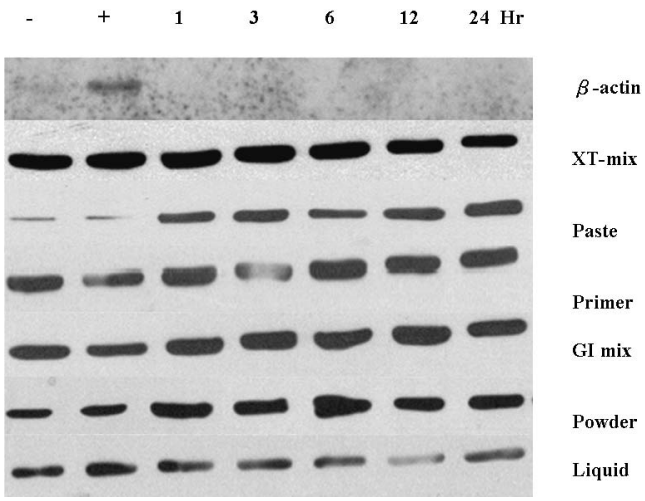

Figure 2. Expression of COX-2 protein level stimulated by Transbond XT paste, Transbond XT primer, Transbond XT mixed, glass ionomer powder, glass ionomer liquid, and glass ionomer mixed groups at different time intervals. All orthodontic adhesives induced COX-2 protein expression in human gingival fibroblasts.

COX-2 mRNA Expression

The expression of COX-2 mRNAs stimulated by the Transbond XT paste, primer, and mixed groups at different time intervals are shown in Figure 3. The level of the COX-2 mRNAs increased by 1.57 and 2.63 fold after being treated with the primer for 1 and 3 hours, respectively. The level of the COX-2 mRNAs increased 1.17, 1.29, and 2.43 fold after treating the paste group for 1, 3, and 6 hours, respectively. The level of the COX-2 mRNAs increased 2.07 and 3.78 fold after treating the mixed resin adhesive group for 1 and 3 hours, respectively (Figure 4).

Expression of COX-2 mRNAs stimulated by the resin hybrid glass ionomer adhesive groups at different time intervals is shown in Figure 5. The level of the COX-2 mRNAs increased 0.89 and 1.28 fold after treating the resin hybrid glass ionomer powder group for 1 and 3 hours, respectively. The level of the COX-2 mRNAs increased 1.24, 1.62, and 1.81 fold after treating the resin hybrid glass ionomer liquid group for 1, 3, and 6 hours, respectively. The level of the COX-2 mRNAs increased 1.89 and 2.41 fold after

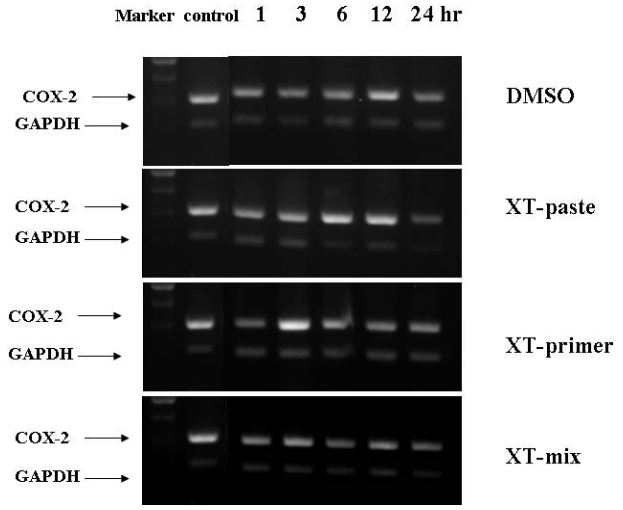

Figure 3. Expression of COX-2 mRNA gene level stimulated by resin base adhesive (Transbond XT) paste, primer, and mixed groups at different time intervals. Control: medium contained with lipopolysacharride and without containing fetal bovine serum; DMSO: dimethysulfoxide.

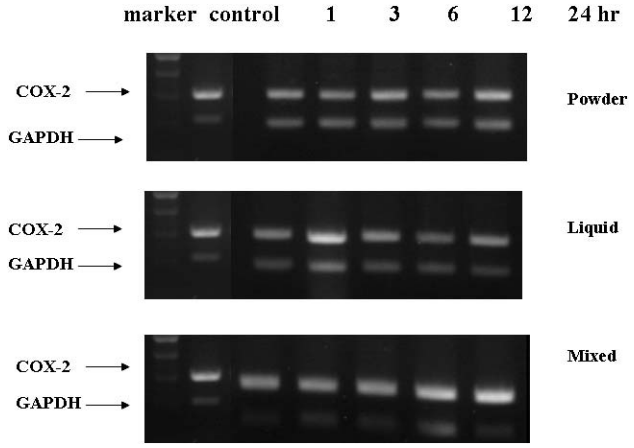

Figure 5. Expression of COX-2 mRNA gene stimulated by resin hybrid glass ionomer base powder, liquid, and mixed groups. Control: medium contained with lipopolysacharride and without containing fetal bovine serum; DMSO: dimethysulfoxide.

treating the mixed resin hybrid glass ionomer adhesive group for 1 and 3 hours, respectively (Figure 6).

DISCUSSION

The resin type or resin hybrid glass ionomer type adhesives showed varied survival rates for human gingival fibroblasts from the present MTT assay. As the treatment time increased, the human gingival fibroblast survival rate decreased (Figure 1). Similar results were found in a study regarding gingival response to dentin bonding agents, which showed that a residual agent can damage periodontal tissue.[18] The reason might be that the chemical composition of the orthodontic adhesive resins is similar to that of dental composites, pit and fissure sealants, and some direct filling resins. These materials usually contain a mixture of high- and low-molecular-weight monomers of methacrylic acid derivatives, such as BIS-GMA (an epoxy derivative of methacrylic acid) and ethyleneglycol dimethacrylate, as well as polymerization inhibitors, catalysts, and accelerators.[19,20] These materials have been shown with various degrees of cellular toxicity.

We found that resin base adhesives and resin hybrid glass ionomer adhesives stimulated COX-2 mRNA and COX-2 protein expression in human gingival fibroblasts. Low levels of release of resin components, such as HEMA or TEGDMA, may pose a risk to normal inflammatory response even though no im-

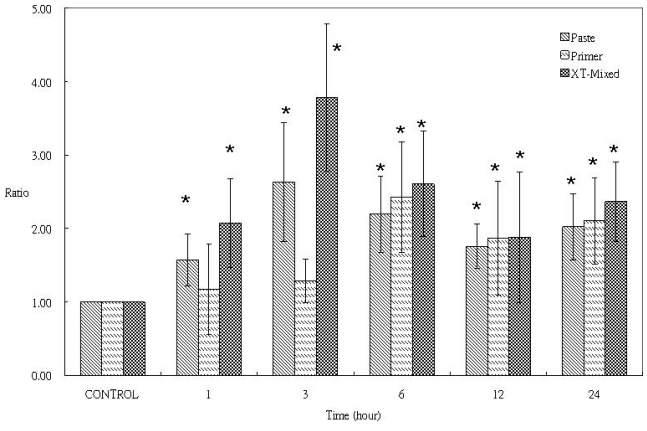

Figure 4. Expression of COX-2 mRNA gene stimulated by resin base adhesive (Transbond XT) paste, primer, and mixed groups at different time intervals. The intensities of the obtained bands were determined with the use of a densitometer (AlphaImager 2000). Tests of differences of the treatments were analyzed using the Tukey test, and a value of $P < .05$ was considered statistically significant.

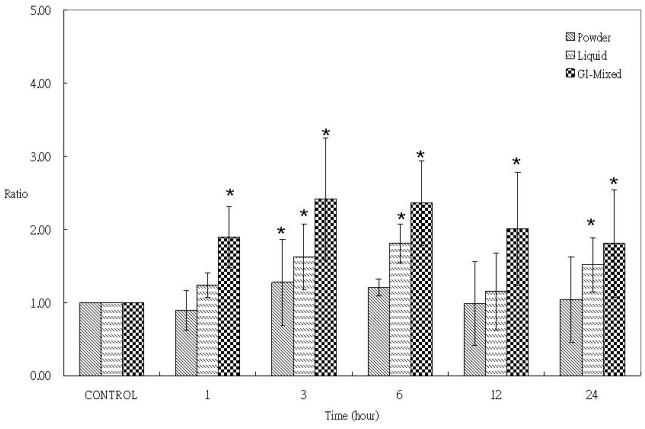

Figure 6. Expression of COX-2 mRNA gene stimulated by resin hybrid glass ionomer base adhesive powder, liquid, and mixed groups at different time intervals. The intensities of the obtained bands were determined with the use of a densitometer (AlphaImager 2000). Tests of differences of the treatments were analyzed by Tukey test, and a value of $P < .05$ was considered statistically significant.

mediate change in cellular function was apparent.[21] This inflammation mechanism was probably controlled by an increase or decrease tumor necrosis factor-alpha (TNF-α) secretion.[21] TNF-α is a cytokine secreted by monocytes that is known to play an important role in inflammatory reactions. In periodontal disease, TNF-α is known to contribute significantly to bone loss and tissue inflammation.[22]

The relationship between TNF-α and COX-2 has been documented as the COX-2 gene promoter region has AP-2 and NF-kB binding sites[23]; both interleukin-1 beta and TNF-α can activate these two transcription factors.[24] TNF-α may play both proinflammatory and protective roles during inflammation by regulation of proinflammatory gene transcripts.[25]

The results also showed the highest level of COX-2 mRNA expression appeared after the adhesives contacted human gingival fibroblasts after 6 hours of treatment (Figures 3 through 6). The COX-2 mRNA expression level of the resin base adhesive was higher than the COX-2 mRNA expression of the resin hybrid glass ionomer adhesive. The COX-2 protein showed high level expression at 24 hours after material contact with human gingival fibroblasts (Figure 2). The paste of the resin base adhesive group and liquid of the resin hybrid glass ionomer adhesive group showed significant levels of COX-2 protein expression (Figure 2). It has been reported that COX-2 expression may play an important role in the pathogenesis of gingival inflammation.[26] The results of our present study showed orthodontic adhesive induced COX-2 expression. Thus, we found that orthodontic adhesives can cause human gingival fibroblast inflammation. The present result differs from the in vivo study of Davidson et al[27] that reported no inflammatory reaction to orthodontic adhesives in the oral mucosa of the Syrian hamster.

When orthodontic patients show gingival inflammation, the residual adhesives on the tooth might be the etiologic factor. The doctors should not always assume that the patient's poor oral hygiene is responsible should be attentive to watch for residual orthodontic adhesive on the teeth. Orthodontists need to avoid gingival tissue contact with orthodontic adhesives and be careful that the overflow adhesive material is cleaned after orthodontic attachments are applied.

CONCLUSIONS

- The resin base and resin hybrid glass ionomer base orthodontic adhesives decreased human gingival fibroblast survival, and increased COX-2 mRNA and COX-2 protein expression.
- To prevent or reduce gingival inflammation in the orthodontic patient, contact of the gingival tissue with adhesives should be avoided.

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