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Differences of multi-species biofilm formation in several orthodontic adhesives

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- ABSTRACT -

Differences of multi-species biofilm formation in several orthodontic adhesives

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Objective: This study investigated the aspects of multi-species biofilm formation on several orthodontic adhesives with different surface characteristics.

Materials and Methods: Multi-species biofilms using 13 bacterial species were grown with three adhesives: composite (Transbond XT, TX), compomer (Transbond Plus, TP) and resin-modified glass ionomer (Multi-Cure, MC). The adhesion levels of *Streptococcus mutans* (Sm), *Streptococcus sobrinus* (Ss), *Porphyromonas gingivalis* (Pg),

Aggregatibacter actinomycetemcomitans (Aa), and total bacteria were determined at day 1 (T1) and day 4 (T2) using real-time polymerase chain reaction. Surface roughness (SR), surface free energy (SFE), and surface texture were analyzed to explain the differences in bacterial compositions among the adhesives. Repeated measures analysis of variance was used to determine time-related differences in bacterial amounts and proportions of each species to total bacteria with respect to adhesive type. The Kruskal-Wallis test was used to determine differences in SR and SFE among the adhesives.

Results: There were no significant differences in adhesion of total bacteria among orthodontic adhesives; however, the adhesion of Sm, Ss, and Pg was higher to MC than the other adhesives. MC also showed higher Sm and Ss proportion to total bacteria than the other adhesives. From T1 to T2, the amount of Sm, Ss, and total bacteria increased, while Pg and Aa decreased. However, proportions of all the tested bacteria to total bacteria were significantly decreased with increased incubation time. MC showed a rougher surface than TX or TP due to the presence of micro-pores and/or flaws. TP had the greatest SFE followed by MC and TX. Interestingly, SR differences were about 10 times greater than SFE differences between the adhesives.

Conclusion: Considering the greater differences in SR than SFE among the adhesives, the rougher surface of MC may cause greater adhesion of Sm, Ss, and Pg.

Keywords : Multi-species biofilm, Orthodontic adhesive, Surface free energy, Surface roughness

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- CONTENTS -

I. INTRODUCTION

The common side effects during fixed orthodontic treatment are enamel demineralization and gingival inflammation[1,2]. The placement of fixed orthodontic appliances promotes biofilm formation, because it impedes access to the tooth surfaces for cleaning and provide retention sites for oral bacteria[3,4]. The oral biofilm is the main cause of infectious oral diseases. Mutans streptococci (MS), *Streptococcus mutans* and *Streptococcus sobrinus* in the biofilm are generally considered the major causative organism of enamel demineralization due to their ability to adhere to the tooth surface and produce acid thorough their metabolism[5]. Periodontal research has reported that *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, which exist in oral biofilm, play an important role in developing the immune-inflammatory response in gingival tissues[6].

Surface characteristics, including surface roughness (SR) and surface free energy (SFE) of biomaterials are reported to influence biofilm formation and the retention on their surface[7,8]. Rough surfaces make biofilm formation and maturation favorable by protecting bacteria from

external forces and increasing adhesion areas[8]. A surface with high SFE was reported to thermodynamically promote bacterial adhesion[8]. Orthodontic adhesives around fixed appliances are the critical site for oral biofilm formation because of their rough surfaces and relatively high SFE[9].

Many investigators have evaluated bacterial adhesion to various orthodontic adhesives, but most studies handled the interaction between orthodontic adhesives with single bacterial species, mainly *S. mutans*[10-16]. However, single-species models are not appropriate to simulate the complex situation of oral biofilm on orthodontic adhesives, because oral bacteria do not exist as independent entities but rather function as a member of integrated microbial communities[17]. In this study, a 13-species biofilm was formed on different orthodontic adhesives and compositional changes in the multi-species biofilm were analyzed using quantitative real-time polymerase chain reaction (PCR). In addition, surface characteristics were analyzed to explain differences in bacterial composition among the adhesives. The purpose of this study was to investigate the aspect of multi-species biofilm formation on several orthodontic adhesives with different surface characteristics. The null hypothesis was that there would be no significant differences in biofilm formation with respect to adhesive type and incubation time.

II. REVIEW OF LITERATURE

1. Biofilm as an etiologic factor of oral disease during fixed orthodontic treatment

1.1 Enamel demineralization

Enamel demineralization during orthodontic treatment is a common side effect during fixed orthodontic treatment[1,2]. This lesion has been major concern of the orthodontists because the lesions are not only unesthetic and unhealthy, but also potentially irreversible[18].

The oral biofilm on the tooth surface is the cause this demineralization. Acidogenic bacteria in the biofilm can produce acid when they metabolize fermentable carbohydrates[19,20]. These acids, especially lactic acid can rapidly dissolve the calcium phosphate of the enamel and result in subsurface enamel porosities[5,21,22]. Among acidogenic bacteria, MS and the lactobacilli group predominantly produce lactic acid and are generally considered the major causative organism of enamel demineralization[20]. Enamel defect caused by demineralization changes light scattering pattern

of the surface and give rise to unesthetic white appearance[1].

The incidence of enamel demineralization during orthodontic treatment is around 60% even with conservative estimation[2]. Although with standard prophylactic measure during the orthodontic treatment, it may turn into actual cavities and progress to severe lesions requiring professional care[23]. Severe lesions requiring professional caries treatment are reported to come up to 15% of patients[23].

1.2. Gingival inflammation

Gingival inflammation, frequently accompanying gingival hyperplasia, is also very common during orthodontic treatment[2]. If the inflammation extended deep into the tissue, it may cause loss of attachment and alveolar bone and is known as periodontitis[6].

Oral biofilm is the primary etiological factor in the development of gingival inflammation and periodontitis[24]. Chronic presence of biofilms around the tooth and its supporting structure, especially gingival pockets result in immune-inflammatory response with their virulence factors[25]. If the host is susceptible, the immune-inflammatory response that develops

in the gingival and periodontal tissues cause destruction of structural components of the periodontium[6].

From the research on the periodontal-disease-associated microflora, *P. gingivalis, Tannerella forsythensis, Treponema denticola* and *A. actinomycetemcomitans* are found to reproducibly associate with the periodontal disease[6].

Various studies on periodontal changes with orthodontic treatment suggested that the pathologic change is only transient and reversible[26,27]. The other studies, however, reported significant attachment loss[28], increase in biofilm pathogenicity and worsened periodontal clinical parameters[29], indicating preventive measure may be required.

2. Effects of surface characteristics on biofilm formation

2.1. Surface roughness (SR)

SR has significant impacts on bacterial adhesion and biofilm formation[7,8,17]. A rough surface is prone to bacterial adhesion and stagnation by increasing the adhesion areas and preventing dislodgement of bacterial colonies[8,9]. SR of specific surface area can be measured using

scanning electron microscopy (SEM) imaging.

Various *in vivo* studies examined the effect of SR on the biofilm formation. Shafagh[30] compared plaque accumulation on cast gold crown with different SR and found that plaque formation occurred at a slower pace in highly polished crown. An *in vivo* study using strips with different SR that are stuck to the labial surface of central incisors of volunteers revealed that surface with low SR delay plaque accumulation[7]. Quirynen et al.[31] evaluated biofilms formed on standard and roughened abutment. As a result, the supragingival plaque of rough abutments had significantly fewer coccoid microorganisms than standard ones, which is indicative of a more mature plaque[31].

Studies using SEM revealed that initial colonization of the bacteria starts from surface irregularities and afterward spreads out from these areas[8,32,33]. This indicates that the initial adhesion of bacteria in supragingival area preferably starts at location that they can be sheltered by shear forces so that a change from reversible to irreversible bonding occurs[8].

Roughening of the surface increases the surface area for adhesion about two to three times[8]. Once the bacteria attach to irregular areas, it may

survive longer because they can be protected against natural removal forces[34] or oral hygiene measure[8]. Moreover, rough surfaces lead to a rapid growth of the biofilm by multiplication of remaining species[17].

2.2. Surface free energy (SFE)

SFE represents the reactivity of a surface and is defined as the work required to increase the area of a substance by one square centimeter[35]. SFE can be calculated by measuring the contact angles between liquids and specific surface[36].

Lifshitz-van der Waals acid-base (LWAB) approach, developed by Fowkes[37], van Oss et al.[38], Good[39] and Chaudhury[40], is useful method to calculate not only SFE but also its polar, dispersive, acid and base component of given solid surface[36]. The formula for solid-liquid interfacial tension is as follows[36]:

$$(1+\cos\Theta)\,\gamma_L^{TOT} = 2(\gamma_S^D\gamma_L^D)^{0.5} + 2(\gamma_L^-\gamma_S^+)^{0.5} + 2(\gamma_S^-\gamma_L^+)^{0.5}$$

Where γ_L and γ_S means liquid's surface tension and solid's surface tension, respectively. Superscript TOT, D, -, and + labels total SFE, dispersive component, Lewis acid component and Lewis base component,

respectively. With a liquid of known SFE components (γ_L^{TOT}, γ_L^D, γ_L^-, and γ_L^+), contact angle Θ can be measured experimentally[36].

Using three liquids including non-polar one, this equation can be readily solved, and SFE components of specific solid surface can be calculated. As for non-polar liquid, Lewis acid and Lewis base component can be neglected ($\gamma_L^- = \gamma_L^+ = 0$), aforementioned formula can be simplified as follows[36]:

$$(1+\cos\Theta)\,\gamma_L^{TOT} = 2(\gamma_S^D \gamma_L^D)^{0.5}$$

When the non-polar liquid's SFE components are given, this can be solved for dispersive component of solid (γ_S^D). With two additional liquids of known SFE components, the other SFE components of solid surface can be readily calculated[36].

Surface with high SFE has been reported to thermodynamically favor the adhesion of bacteria[8,17]. In the oral cavity, hard surfaces are coated by pellicle and it lowers SFE[41]. Therefore, impact of SFE may be reduced due to homogenizing effect[42-44] of the coating. Various studies reported that pellicle coating resulted in general reduction of bacterial adhesion irrespective of the surficial SFE[41,42,45-47].

Although the impact of SFE is decreased after the pellicle coating, the thermodynamic approach still remains of value. Pratt-Terpstra et al.[45] evaluated the adhesion of streptococcal strains on the surface with various SFEs. They reported that although the degree of bacterial adhesion decreased after protein film coating, the order of adhesion amount was similar to that in the case of uncoated surface[45]. A study about SFE characteristics of various denture base materials also revealed adhesive properties were influenced by the surface[48]. Those results indicate that SFE properties are transferred thought the acquired pellicle layer[17,45,48].

Various *in vivo* or animal studies supported that the surface with higher SFE are more prone to biofilm formation. Glantz[49] investigated the relationship between SFE and amounts of accumulated dental biofilm formed on the various surfaces mounted on the fixed partial denture. In this *in vivo* study, positive correlation between SFE and the weight of accumulated dental biofilm was found[49]. van Dijk et al.[44] attached various facings on crown of beagle dogs to find the relationship between SFE and bacterial adhesion and revealed more micro-organisms adhered to substrata with high SFE than one with low SFE. Quirynen et al.[7,50] analyzed *in vivo* dental biofilm accumulation on strips made of fluorethylenepropylene (FEP) and cellulose acetate (CA). Less biofilm was

accumulated on strips made of FEP than ones made of CA because of their lower SFE[7,50]. Rølla et al.[51] demonstrated that the application of a silicone oil to teeth reduces biofilm formation because of lowered SFE.

2.3. Surface chemical composition

Surface chemistry is also known to influence bacterial adhesion and proliferation in a manner depending on material hydrophobicity and charge[52]. Tegoulia and Cooper investigated *Staphylococcus aureus* adhesion on self-assembled monolayers (SAMs) terminated with methyl, hydrohylic, carboxylic acid and tri (ethylene oxide) groups and revealed that adhesion on the carboxylic- and methyl-terminated SAMs were much higher than ethylene oxide-bearing surfaces[53].

Composite, compomer and resin-modified glass ionomer (RMGI) are widely used orthodontic adhesives. Composites are composed of three major components: resin matrix that forms a continuous phase and binds the filler particles; filler particles dispersed in the matrix which reinforces resin matrix; and coupling agent which act as bonding agent that promotes adhesion between filler and resin matrix[54]. Most composite use a blend of aromatic and/or aliphatic dimethacrylate monomers such as bisphenol A

diglycidyl ether dimethacrylate (bis-GMA), triethylene glycol dimethacrylate (TEGDMA) and unrethane dimethacrylate (UDMA) as resin matrix[54]. In many cases, filler particles are produced by grinding or milling quartz or glasses to produce silica particles[54]. Fillers are bonded to the resin matrix with coupling agents and in most case, it is organosilanes including ɣ-methacryloxypropyl trimethoxysilane[54].

RMGI is modified glass ionomer that originally hardens with acid-base reaction between fluoroaluminosilicate glass powder and polyacrylic acid[54]. It incorporates a cross-linking agent and part of the polyacrylic acid is replaced with hydrophilic monomers[54]. Its powder component consists of ion-leachable fluoroaluminosilicate glass particles and initiators for light curing and/or chemical curing[54]. Liquid component usually contains water, polyacrylic acid like itaconic, maleic, or tricarboxylic acids and polyacrylic acid modified with methacrylate and hydroxyethyl methacrylate (HEMA) monomers[54]. It is cured by polymerization of methacrylate groups initially, and followed by slow acid-base reaction[54]. It has caries preventive effect because it released fluoride[55] during the setting process with the acid etches the surface of the fluoroaluminosilicate glass particles[54]. However, it was reported that the fluoride release from RMGI rapidly decreases due to wash out effect[56].

Compomer is resin-based composite material containing silicate glass filler particles and methacrylate and acidic monomers as matrices[54]. It undergoes an acid-base reaction in aqueous environment and release fluoride during the process[54]. It has structural and physical properties similar to those of composite[54].

3. Previous studies on bacterial adhesion to orthodontic adhesives

Many studies have evaluated bacterial adhesion to various orthodontic adhesives. Hallgren et al.[10] compared cariogenic microflora including MS and *Lactobacilli* in biofilm adjacent to orthodontic bracket bonded with a glass ionomer cement and composite using the split mouth design. Both of bacteria showed a tendency to colonize higher to composite than glass ionomer cement (GIC)[10]. After one month of treatment, the proportion of MS to the total bacteria was significantly higher in composite group than a GIC group[10]. They suggested that a GIC may develop less cariogenic microflora[10].

Blunden et al.[11] examined the adhesion of *S. mutans* to the composites and a GIC. They assessed colonization of bacterium to adhesives by

weight gain and SEM[11]. The results revealed a stronger positive correlation between weight gain and SFE than that of between weight gain and SR[11]. They postulated that SFE has a greater influence than SR on bacterial colonization[11].

Ahn et al.[13] analyzed the SR and SFE of orthodontic adhesives and their effects on the adhesion of MS to adhesives. They incubated each material with saliva or phosphate buffered saline (PBS) solution to investigate the influence of saliva coating on bacterial adhesion[13]. The results demonstrated that SFE of RMGIs was significantly higher than composites, whereas SR of various adhesives was relatively uniform[13]. MS adhered to RMGI significantly more than composites and a compomer, and positive correlation was found between SFE and MS adhesion[13]. Saliva coating did not significantly influence MS adhesion[13]. Therefore, the researchers concluded that MS adhesion to adhesives is significantly influenced by SFE characteristics rather than saliva coating[13].

Mei et al.[14] investigated that adhesion forces between Streptococcus (*Streptococcus sanguinis* and *S. mutans*) and composites with different SR using atomic force microscopy with or without salivary conditioning film. Salivary conditioning films significantly lowered the SR and adhesion

forces[14]. Rougher surfaces made adhesion forces stronger, irrespective of composite type or bacterial strain[14]. And the influence of SR was stronger to adhesion forces of *S. sanguinis* than that of *S. mutans*[14].

Various studies including aforementioned ones have evaluated bacterial adhesion to various orthodontic adhesives. However, difference among type of adhesive is contradictory with respect to studies and effect of surface characteristics on bacterial adhesion is not clear. Moreover, most studies adapted simple model investigating the interaction between orthodontic adhesives with single bacterial species, mainly *S. mutans*[10-16].

4. Multi-species biofilm model using CDC biofilm reactor

Bacterial consortiums composed of 9 to 10 species has been suggested and modified in previous studies[57-65], with regard to their major prevalence in oral biofilm, range of metabolic activities, relevance in health and disease, and ease of isolation and identification[57,63]. Although there was slight modification, they were mainly composed of following ten species[57-64]: *Actinomyces naeslundii, Prevotella nigrescens, Fusobacterium nucleatum, Lactobacillus casei, Neisseria subflava, Streptococcus oralis, Streptococcus gordonii, S. mutans, Veillonella dispar,* and *P. gingivalis*.

Shu et al.[65] adopted this consortium (They replaced *S. gordonii* to *Streptococcus salivarius*) and cultivated multi-species oral biofilm using constant-depth film fermenter using modified McBain medium (MBM). The medium contained 2.5 g/L porcine gastric mucin, 2 g/L proteose peptone, 2.5 g/L KCl, 1 g/L yeast extract, 1 g/L trypticase peptone, 0.1 g/L cysteine hydrochloride, 0.001 g/L hemin, 10 mM glucose, and 10 mM urea[65]. Among these, porcine gastric mucin, proteose peptone, KCl, yeast extract, trypticase peptone, cysteine hydrochloride, and hemin are used as ingredients of artificial saliva[66,67]. Therefore, this medium can supply nutrients to bacteria and at the same time, act as artificial saliva. A previous study used McBain medium as artificial saliva suggested that the medium can be used as equivalent to human saliva because it produces a medium-derived pellicle[68].

A CDC biofilm reactor (CBR), developed by Donlan et al.[69] is used to provide reproducible biofilm samples for the evaluation of antimicrobial agents and surface materials[70]. Top of CBR can hold eight independent rods that can be immersed in growth medium, and each rod has three disk hosing. A study demonstrated that total 24 position of disk has no significant difference[71]. Because disks can be made with various materials, evaluation for diverse surface is possible at the same time. As nutrients

are continuously pumped into and flow out of the reactor at the same rate, steady state condition can be achieved. With the magnetic stirrer, CBR can exert controllable shear force to the surface and form dynamic environment.

In comparison with previous fermenter, CBR has several advantages. Because each rod can be removed intermittently, the reactor allows evaluation of time-related microbial changes. In addition, CBR provides dynamic condition rather than static one. As the fluid is pumped in and out with the same rate and mixed continuously, steady state condition without concentration gradient can be assumed. Furthermore, controllable shearing force from stirring simulates fluid shear in the oral cavity. Previous research used CBR to grow a standard biofilm for diverse application over time[69,71].

III. MATERIALS AND METHODS

1. Preparation of orthodontic adhesive specimens

Three orthodontic adhesives were used in this study: composite (Transbond XT, 3M Unitek, Monrovia, CA, USA) (TX); compomer (Transbond Plus, 3M Unitek) (TP); and resin-modified glass ionomer cement (Multi-Cure, 3M Unitek) (MC). For each adhesive, a total of 61 adhesive disks were prepared with Teflon templates (a diameter of 12.7 mm and thickness of 3.0 mm): 20 for biofilm experiments, 10 for measuring SR, 30 for analyzing SFE, and one for SEM.

2. Bacterial strains and culture methods

We used 13-species bacterial consortium containing *S. mutans* ATCC 700610, *S. sobrinus* ATCC 27607, *S. sanguinis* CCUG 17826, *S. salivarius* CCUG 50207, *S. oralis* ATCC 9811, *A. naeslundii* KCOM 1472, *Lactobacillus rhamnosus* ATCC 7469, *V. dispar* KCOM 1864, *N. subflava* ATCC 49275. *F. nucleatum* ATCC 10953, *P. nigrescens* ATCC 33563, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718

because of their major prevalence in oral biofilms, range of metabolic activities, relevance in health and disease, and ease of isolation and identification[57,63].

Each bacterial strain was individually grown to mid-exponential phase. *S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. salivarius*, *S. oralis*, *A. naeslundii*, *L. rhamnosus*, *V. dispar*, and *N. subflava* were grown in a brain heart infusion (BHI, Becton Dickinson, Sparks, MD, USA) medium at 37°C with 5% CO_2. *F. nucleatum*, *P. nigrescens*, and *P. gingivalis* were anaerobically grown in a tryptic soy agar (Becton Dickinson) medium supplemented with 10 μg/mL vitamin K, 5 μg/mL hemin and 5% sheep blood at 37°C for 7 days. These species were subcultured in BHI medium supplemented with 10 μg/mL vitamin K, 5 μg/mL hemin and grown to mid-exponential phase anaerobically at 37°C. *A. actinomycetemcomitans* was grown in a BHI medium at 37°C in an anaerobic atmosphere.

3. Multi-species biofilm formation with CDC biofilm reactor

For multi-species biofilm formation, we used MBM containing 2.5 g/L porcine gastric mucin, 2 g/L proteose peptone, 2.5 g/L KCl, 1 g/L yeast extract, 1 g/L trypticase peptone, 0.1 g/L cysteine hydrochloride, 0.001

g/L hemin, 10 mM glucose, and 10 mM urea[70,72]. The medium includes components of artificial saliva in order to allow the maintenance of complex and stable salivary ecology. In particular, mucin which occupies about 26% of salivary proteins in natural saliva, can cover the adhesive surfaces similar to natural saliva[68].

A CDC biofilm reactor (BioSurface Technologies, Bozeman, MT, USA) was used as a device for cultivating multi-species biofilms[69]. The lid of the biofilm reactor can support eight independent rods which individually hold three adhesive disks. Three different adhesive disks were randomly inserted into each rod before experimentation. After the rods with adhesive disks, the equipment, and MBM were sterilized, the reactor was set on a hot stir plate set at 37°C and a rotation speed of 60 rpm. Mixture of cell culture listed above was added to the biofilm reactor (3.5 mL, 1% of the reactor volume) and a continuous flow of MBM was then flushed through the reactor at a rate 100 mL/h during the experiment.

4. Biofilm removal and DNA extraction

To analyze time-related differences in biofilm formation among the three adhesives, two rods containing six adhesive disks (two sets of three

adhesive disks) were removed from the reactor at two time points: days 1 (T1) and 4 (T2). Each disk was carefully removed and transferred into a round tube before washing two times with 1.0 mL PBS (pH = 7.4) to remove unbound bacteria. The biofilm was then detached from each disk by sonication with three 30-sec pulses and 30-sec intermittent cooling stages in a chilled ice box. After removing the disk, the suspension was centrifuged at 13,000 rpm for 10 min and washed twice with 1.0 mL PBS.

Bacterial chromosomal DNA was extracted using a CellEase Bacteria II Genomic DNA Extraction Kit (Biocosm, Osaka, Japan) according to the manufacturer's instructions. A NanoVue spectrophotometer (General Electric Healthcare Life Sciences, Pittsburgh, PA, USA) was used to assess the quality of the extracted DNA after preparation.

Known specific PCR primers that amplify the dextranase genes of *S. mutans* and *S. sobrinus* were designed from the *gtfB*, *gtfU* genes, respectively[73]. The PCR primer of *P. gingivalis* was designed from the 16S rRNA gene, and the primer of *A. actinomycetemcomitans* was based on the *rpoB* gene of RNA polymerase β subunit gene[74]. A conserved sequence in the 16S rRNA was selected to quantify the numbers of total bacteria[73] (Table 1). All primers were commercially synthesized (Bioneer, Daejeon,

Korea).

DNA was extracted from *S. mutans* ATCC 700610, *S sobrinus* ATCC 27607, *P. gingivalis* KCOM 2797, and *A. actinomycetemcomitans* ATCC 43718 using a G-spin Genomic DNA Extraction kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. The DNA standard curve was obtained from known amounts of purified PCR product. PCR products were isolated from agarose gels using a QIAquick Gel Extraction Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instruction. DNA concentration was estimated by absorbance at 260 nm and series of 10-fold dilutions ranging from 10 to 10^7 copies were prepared for standard curves as previously described[75]. The amount of bacterial DNA in the samples was estimated from the standard curve.

5. Bacterial quantification with real-time PCR

Real-time PCR was performed using the iQ5 system (Bio-Rad, Hercules, CA, USA). The reaction mixtures contained 2 μL of purified DNA from the disk samples, 100 pmol of primer, and 10 μL of 2x iQ SYBR Green Supermix (Bio-Rad). Distilled water was added to a final volume of 20 μL.

The cycling conditions used were as follows: for universal primers, and specific primers for *S. mutans* and *S. sobrinus*, initial denaturation for 30 sec at 94°C, 40 cycles of denaturation for 20 sec at 95°C, primer annealing for 45 sec at 60°C, and extension for 10 sec at 60°C; for *P. gingivalis* primers, initial denaturation for 1 min at 95°C, 40 cycles of denaturation for 5 sec at 95°C, primer annealing for 15 sec at 61°C, and extension for 33 sec at 72°C, and final extension for 10 min at 72°C; and for *A. actinomycetemcomitans* primers, initial denaturation for 10 min at 95°C, 40 cycles of denaturation for 10 sec at 95°C, primer annealing and extension for 30 sec at 72°C. All data were analyzed using iQ5 Optical System Software (Bio-Rad). All the experiments for quantifying bacterial levels were performed in duplicate and independently repeated five times.

6. Surface characteristics analysis of orthodontic adhesives

The SR was analyzed using confocal laser scanning microscope (LSM 5 Pascal, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). This allows for calculation of the arithmetic mean SR from a mean plane within the sampling area (230 μm X 230 μm X 30 μm). The measurements were performed on the ten specimens of each adhesive.

The SFE and its component parts, the nonpolar (γ^{LW}) and polar acid/base component (γ^{AB}), which is further divided into acid (γ^{-}) and base (γ^{+}) components, were measured by the sessile drop method using deionized distilled water, 1-bromonaphthalene, and formamide as probe liquids as previously described[9].

To examine the surface texture of the orthodontic adhesives, SEM was used. Each surface was observed with a magnification set at 3000X using a S-4700 microscope (Hitachi, Tokyo, Japan).

7. Statistical analysis

Repeated measures analysis of variance using the Bonferroni correction was used to determine the time-related differences in bacterial amounts and proportions of each species to total bacteria with respect to adhesive type. The Kruskal-Wallis test was used to determine the differences in SR and SFE characteristics among the three adhesive groups. For all analyses, $\alpha < 0.05$ was considered statistically significant.

IV. RESULTS

During primer specificity testing, bacterial genomic DNA showed a specific DNA band around 160 bp with the universal primer set. For the specific primers, only the target bacteria produced a single DNA fragment corresponding their specific primers and amplified DNA was not detected in other species (data not shown).

Changes in levels of target bacteria with respect to incubation time and adhesive type are shown in Tables 2 and 3. There were no significant differences in the adhesion of total bacteria among the adhesives (TX = TP = MC, Table 2). However, composition of oral pathogens in the biofilm was significantly different among the adhesives. The adhesion of MS and *P. gingivalis* were higher with MC relative to the other adhesives (TX = TP < MC, Table 2, and Figures 1A and 1B). MC also showed higher MS proportion to total bacteria than the other adhesives (Table 3).

Incubation time significantly influenced the composition of oral pathogens in biofilms. The amount of total bacteria and MS increased from T1 to T2 (T1 < T2), while *P. gingivalis* and *A. actinomycetemcomitans*

significantly decreased from T1 to T2 (T1 > T2, Table 2). Proportions of all the test bacteria to total bacteria were significantly decreased with increased incubation time (T1 > T2, Table 3).

The proportion of *S. mutans* to the total bacteria showed significant interaction between incubation time and adhesive type (P = 0.033). From T1 to T2, *S. mutans* to total bacteria ratio decreased in all adhesives, but the adhesion of *S. mutans* to MC more sharply decreased than the others (Table 3).

There were significant differences in SR among the adhesives. The order of SR was, from the lowest to highest, TX, TP, and MC (TX < TP < MC, Table 4). However, the difference between TP and MC (0.15 μm) was higher than that between TX and TP (0.03 μm). SEM images showed that adhesive surface textures were consistent with the SR data. MC had larger micro-pores and/or flaws on its surface relative to TX and TP (Figure 2).

There were significant differences in SFE and its components among the adhesives, but the pattern was different from SR. TP had the greatest SFE and polar, acid and base component followed by MC and TX (TX < MC < TP, Table 4). On the contrary, TX had the greatest dispersive component followed by TP and MC (MC < TP < TX, Table 4).

V. DISCUSSION

Oral biofilms are the main etiologic factor for enamel demineralization and gingival inflammation during orthodontic treatment[5,24,76]. Because adhesive remnants around the orthodontic appliances are supposed to be the critical site for biofilm formation[9], many researchers have evaluated the adhesion of bacteria to the surface of adhesives[10-16]. However, most biofilm studies have focused on cariogenic bacteria rather than periodontopathogens[10,11,13-16] and have investigated the adhesion of single species of pathogenic bacteria to orthodontic adhesives[10-16] rather than multi-species biofilms. In this study, we used a multi-species biofilm model including 13 representative species to investigate compositional changes of oral pathogens in biofilms formed on orthodontic adhesives with respect to surface characteristics.

This study showed that composition of oral pathogens in the multi-species biofilm model was significantly influenced by adhesive type. The adhesion level of total bacteria was not significantly different (TX = TP = MC, Table 2), but the composition of oral pathogens within the biofilm was significantly different among the three adhesives. MC significantly

enhanced adherence of MS relative to the other two adhesives (TX = TP < MC, Table 2, and Figures 1A and 1B). The proportions of MS to total bacteria were also significantly higher in MC (TX = TP < MC for *S. mutans* and TX < TP < MC for *S. sobrinus*, Table 3). In addition, the adhesion of *P. gingivalis* to MC was significantly higher than the others (TX = TP < MC, Table 2, and Figures 1A and 1B). These findings suggest MC offer favorable environments for enamel demineralization and gingival inflammation by providing favorable niches for oral pathogens. This is partly consistent with the findings of previous single-species adhesion studies showing that MS adhered to RMGIs significantly more than to composites or compomers[13,18].

The composition of oral pathogens in biofilms was also significantly influenced by incubation time. MS adhesion to the adhesives significantly increased with extended incubation time (T1 < T2, Table 2). However, their proportions to total bacteria significantly decreased with time (T1 > T2, Table 3). This can be explained by the interaction between different oral species which adhere to underlying surfaces, with initial or early colonizers, intermediate, and late colonizers[77]. Because MS are members of the early colonizers[77], increased MS adhesion during the incubation period may promote adhesion of other intermediate or late colonizers by

providing binding receptors and interactions with each other[77,78]. As a result, the adhesion level of total bacteria may be greater than that of MS alone, which may decrease relative proportions of MS compared to total bacteria.

The adhesion of periodontopathogens including *P. gingivalis* and *A. actinomycetemcomitans* as well as their proportion to total bacteria significantly decreased with extended incubation time (T1 > T2, Tables 2 and 3). Because the biofilm formation was operated in an aerobic condition in this study, adhesion of periodontopathogens may decrease with extended incubation time due to their anaerobic nature. This is partly supported by the fact the facultative anaerobe, *A. actinomycetemcomitans* grew better than obligate anaerobe, *P. gingivalis* (Tables 2 and 3).

A significant interaction between incubation time and adhesive type was found in the proportion of *S. mutans* to the total bacteria. Between T1 and T2, *S. mutans* to total bacteria ratio in MC decreased more steeply than TP and TX (Table 3). This may be due to the rougher surfaces of MC. MC may efficiently shelter colonization and growth of biofilm on their surfaces during the experiment, compared to TX or TP. Therefore, rapid adhesion of bacteria other than *S. mutans* may make *S. mutans* to total bacteria ratio

lower in MC than other adhesives as incubation time increase.

In this study, surface characteristics were analyzed to explain the reason for differences in biofilm formations among the adhesives. The results showed that MC had higher SR than other two adhesives (TX < TP < MC, Table 4) due to the presence of larger micro-pores and/or flaws on the surfaces (Figure 2). In contrast to SR, SFE was highest in TP than MC and TX (TX < MC < TP) and other SFE components including γ^{AB}, γ^{-}, and γ^{+} of MC were intermediate between TX and TP (TX < MC < TP, Table 4). For γ^{LW}, MC was the lowest (MC < TP < TX, Table 4).

Significantly higher SFE and its components except γ^{LW} of MC and TP than TX might be mainly due to difference in chemical compositions[9]. RMGI and compomer release ions including fluoride during setting process as a result of reaction between acid and glass fillers[56]. Their setting initiated with light-activated polymerization followed by an acid-base reaction from the absorption of water[56]. The dynamic reaction processes on the surface of RMGI and compomer might be associated with their high SFE and its components.

Higher SFE and its components of TP than MC might be due to their difference in degree of conversion. A previous research reported that

RMGI showed significantly higher degree of conversion and acid neutralization extent than those of compomer[79]. Whereas degree of conversion of RMGI reached over 97% at 150 sec after the start of light exposure, that of compomer was around 60% after 50,000 sec[79]. By 50,000 sec, neutralization extents of RMGI and compomer were about 0.41 and 0.016 times that of the glass ionomer cement, respectively[79]. Therefore, it can be postulated that significantly more residual acidic group might be left on the surface of compomer than that of RMGI. More acidic surface of TP might lead higher SFE and its components.

Aforementioned difference in surface chemical composition also might lead compositional differences in biofilms. On the contrary to composite, RMGI and compomer partially set by acid-base reaction. Because not all of the active groups are reacted and became inert during setting process[79], surface of RMGI and compomer might be acidic, especially at the initial phase of biofilm formation. Therefore, surface of TP and MC might be more favorable for aciduric bacteria including MS to colonize at the initial stage of biofilm formation.

A previous study reported that γ^{LW}, γ^{AB}, and γ^{+} are positively related to MS adhesion[13]. This relationship may be due to the important role of polar

and van der Waals interactions in initial adhesion of bacteria[80,81]. In this study, however, adhesion of MS was higher in MC relative to TX or TP, in spite of its lower SFE characteristics than TX and TP.

The SR of MC is about 115% and 180% greater than those of TP and TX, respectively, while the SFE of TP was only about 8% and 16% greater than MC and TX, respectively. Considering amount of differences in bacterial level with respective adhesive type, SR had dominant effect on adhesion of MS to adhesives because differences in SR between MC and other adhesives are much greater than those of SFE and its components. Although direct comparison is not possible, our findings are similar to those of previous studies reporting SR is a governing factor over the influence of SFE determining the formation of biofilm[8,82]. The reason can be explained in several ways.

Although SFE and its components have impacts on initial adhesion of microorganism[8,17], they may have more influence on the initial colonizers than on the late colonizers. For late colonizers including periodontopathogens (*P. gingivalis* and *A. actionmycetemcomitans* in this sutdy), inter-bacterial communication with pre-formed biofilm may be more important than SFE characteristics. Busscher et al.[83] suggested that

the differences between SFE characteristics rapidly disappear once the bacteria are allowed to start to grow into a mature biofilm, and this report is supported by our findings. In addition, the effects of SFE may be smaller than expected in an aqueous condition. During our preliminary study, water contact angles of the MBM coated adhesives were not significantly different among the three adhesives (data not shown). These findings are consistent with previous studies which have shown that differences in SFE between underlying materials become similar after saliva-coating[42,84].

After the initial adhesion of early colonizers, SR seems to play a leading role in biofilm formation with time[17]. Surfaces with higher SR can protect further bacterial colonization and/or accumulation against shear forces better than those with lower SR and eventually help to maintain firm inter-bacterial interactions or binding between bacteria and surfaces[8,17]. In this study, continuous shear force was exerted to simulate oral environment, which may have resulted SR had greater effects than SFE.

This study showed that both cariogenic and periodontopathic bacteria adhere to orthodontic adhesives. The adhesion and biofilm formation of MS can produce organic acid and cause enamel demineralization near the tooth surface. Although periodontopathogens showed lower adhesion

patterns than MS (Table 3), the results of the present study demonstrated that considerable number of periodontopathogens ranging from 10^2 - 10^4 remained adhered to the orthodontic adhesives. Without proper prophylaxis, periodontopathogens may release from the biofilms around the adhesives and invade periodontal pockets, specifically in the mandibular incisor and/or molar area where the distance between the orthodontic appliance and the gingiva is relatively close. These findings suggest that biofilms around orthodontic adhesives should be considered as one of the risk factors for enamel demineralization and/or gingival inflammation during orthodontic treatment.

This study has the following limitations. We evaluated differences in biofilm formed on various orthodontic adhesives using a multi-species biofilm model to simulate intraoral environment. However, real oral cavity is a way more complex as a habitat of over 750 bacterial species[85]. We also tried to exert continuous shear force to the surface to mimic natural shear force, but it is intermittent in real situation. Finally, the causal relationship between surface characteristics and biofilm formation is unclear because of some uncontrolled variables. Further *in vivo* studies will be needed to investigate the effects of surface characteristics on multi-species biofilm formation.

In spite of aforementioned limitations, the results of the present study suggest that MC, a type of RMGI may provide favorable environments for MS and *P. gingivalis* adhesion, possibly due to its high SR. Although RMGI releases fluoride, the fluoride release from RMGI rapidly decreases due to wash out[56]. Considering both of bacterial adhesion and fluoride release ability and because same type of adhesive has similar surface characteristics[9], this *in vitro* study suggests that compomer is better for an orthodontic adhesive than RMGI in patients with poor oral hygiene.

VI. CONCLUSIONS

This study was performed to investigate the differences in multi-species biofilm formation on three types of orthodontic adhesives. This study showed that composition of oral pathogens in biofilms on orthodontic adhesives significantly differs according to adhesive type and incubation time. Therefore, the null hypothesis of our study was rejected. Adhesion of MS increased with time, whereas the adhesion of periodontopathogens decreased. In particular, MC showed more cariogenic biofilm composition than the other adhesives with extended incubation time, possibly due to its surface chemical composition and higher SR than TX or TP. Despite limitations, our multi-species biofilm model suggests that the use of RMGI adhesive should be carefully considered in orthodontic patients with poor oral hygiene.

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TABLES

Table 1. Specific primers used in this study.

Primer	Sequence (5'- to -3')	Amplicon size (bp)
Universal		
Forward	TGGAGCATGTGGTTTAATTCGA	160
Reverse	TGCGGGACTTAACCCAACA	
Streptococcus mutans		
Forward	CTACACTTTCGGGTGGCTTG	261
Reverse	GAAGCTTTTCACCATTAGAAGCTG	
Streptococcus sobrinus		
Forward	AAAACATTGGGTTACGATTGCG	156
Reverse	CGTCATTGGTAGTAGCCTGA	
Porphyromonas gingivalis		
Forward	TGCAACTTGCCTTACAGAGGG	344
Reverse	ACTCGTATCGCCCGTTATTC	
Aggregatibacter actinomycetemcomitans		
Forward	GGCGAGCCTGTATTTGATGTGCG	113
Reverse	GTGCCCGGTGCTGCGTCTTTG	

Table 2. The levels of bacteria in multi-species biofilm on various orthodontic adhesives at each time point.

	Day 1 (T1)	Day 4 (T2)	Significance†		*P* value for interaction
			Time	Adhesive	
Total bacteria (Log_{10}/cm^2)					
TX[a]	6.89 ± 0.28	7.81 ± 0.31			
TP[b]	6.86 ± 0.40	7.76 ± 0.35	T1 < T2	TX = TP = MC	0.936
MC[c]	6.96 ± 0.36	7.89 ± 0.31			
Streptococcus mutans (Log_{10}/cm^2)					
TX	4.18 ± 0.45	4.57 ± 0.26			
TP	4.26 ± 0.26	4.64 ± 0.25	T1 < T2	TX = TP < MC	0.866
MC	4.53 ± 0.27	4.89 ± 0.22			
Streptococcus sobrinus (Log_{10}/cm^2)					
TX	4.13 ± 0.29	4.68 ± 0.28			
TP	4.29 ± 0.31	4.73 ± 0.17	T1 < T2	TX = TP < MC	0.537
MC	4.54 ± 0.21	5.05 ± 0.20			
Porphyromonas gingivalis (Log_{10}/cm^2)					
TX	3.35 ± 0.62	2.91 ± 0.83			
TP	3.29 ± 0.79	3.06 ± 0.82	T1 > T2	TX = TP < MC	0.111
MC	3.55 ± 0.75	3.55 ± 0.80			
Aggregatibacter actinomycetemcomitans (Log_{10}/cm^2)					
TX	4.43 ± 0.22	4.34 ± 0.23			
TP	4.51 ± 0.28	4.28 ± 0.40	T1 > T2	TX = TP = MC	0.174
MC	4.57 ± 0.39	4.31 ± 0.64			

† Two-way repeated measures ANOVA was used to determine significant differences between the two time points using the Bonferroni correction at a significant level of $\alpha < 0.05$: [a], Transbond XT; [b], Transbond Plus; [c], Multi-Cure.

Table 3. The proportions of bacteria in multi-species biofilm on various orthodontic adhesives at each time point.

	Day 1 (T1)	Day 4 (T2)	Significance†		*P* value for interaction
			Time	Adhesive	
Streptococcus mutans/total bacteria (%)					
TX[a]	0.24 ± 0.14	0.07 ± 0.04			
TP[b]	0.29 ± 0.15	0.09 ± 0.07	T1 > T2	TX = TP < MC	0.033
MC[c]	0.44 ± 0.20	0.11 ± 0.06			
Streptococcus sobrinus/total bacteria (%)					
TX	0.19 ± 0.08	0.08 ± 0.04			
TP	0.31 ± 0.18	0.11 ± 0.08	T1 > T2	TX < TP < MC	0.075
MC	0.42 ± 0.19	0.19 ± 0.13			
Porphyromonas gingivalis/total bacteria (%)					
TX	0.06 ± 0.05	0.01 ± 0.01			
TP	0.07 ± 0.08	0.01 ± 0.01	T1 > T2	TX = TP = MC	0.168
MC	0.12 ± 0.14	0.01 ± 0.01			
Aggregatibacter actinomycetemcomitans/total bacteria (%)					
TX	0.47 ± 0.28	0.06 ± 0.07			
TP	0.71 ± 0.68	0.10 ± 0.16	T1 > T2	TX = TP = MC	0.168
MC	0.78 ± 0.91	0.10 ± 0.13			

† Two-way repeated measures ANOVA was used to determine significant differences between the two time points using the Bonferroni correction at a significant level of $\alpha < 0.05$: [a], Transbond XT; [b], Transbond Plus; [c], Multi-Cure.

Table 4. Surface roughness (SR), surface free energy (SFE), and surface free energy components (dispersive, polar, acid, and base components) of orthodontic adhesives used in this study.

	Orthodontic adhesives			Multiple comparisons†
	TX[a]	TP[b]	MC[c]	
SR (μm)	0.10 ± 0.01	0.13 ± 002	0.28 ± 0.07	TX < TP < MC
SFE (mJ/m)	46.35 ± 2.15	53.96 ± 0.66	50.02 ± 1.82	TX < MC < TP
Dispersive (mJ/m)	42.88 ± 0.56	40.41 ± 0.93	39.47 ± 0.47	MC < TP < TX
Polar (mJ/m)	3.47 ± 2.64	13.55 ± 1.58	10.56 ± 2.27	TX < MC < TP
Acid (mJ/m)	0.28 ± 0.20	1.73 ± 0.24	1.25 ± 0.48	TX < MC < TP
Base (mJ/m)	17.39 ± 1.32	26.59 ± 2.62	23.15 ± 0.77	TX < MC < TP

† The Kruskal-Wallis test was used to determine the differences among the three groups and multiple comparisons using the Mann-Whitney tests with the Bonferroni correction at a significant level of $\alpha < 0.05$: [a], Transbond XT; [b], Transbond Plus; [c], Multi-Cure.

FIGURES

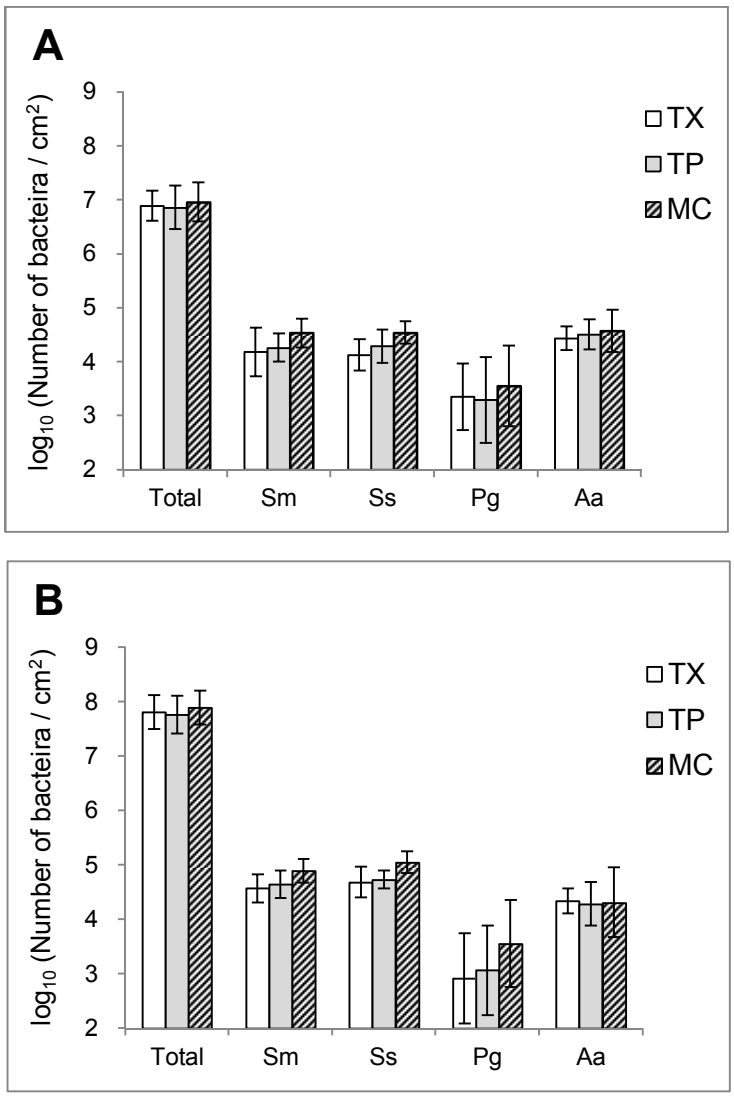

Figure 1. Changes in level of bacteria in biofilms formed on adhesives at (A) Day 1 (T1) and (B) Day 4 (T2); Total: total bacteria; Sm: *Streptococcus mutans*; Ss: *Streptococcus sobrinus*; Pg: *Porphyromonas gingivalis*; Aa: *Aggregatibacter actinomycetemcomitans*.

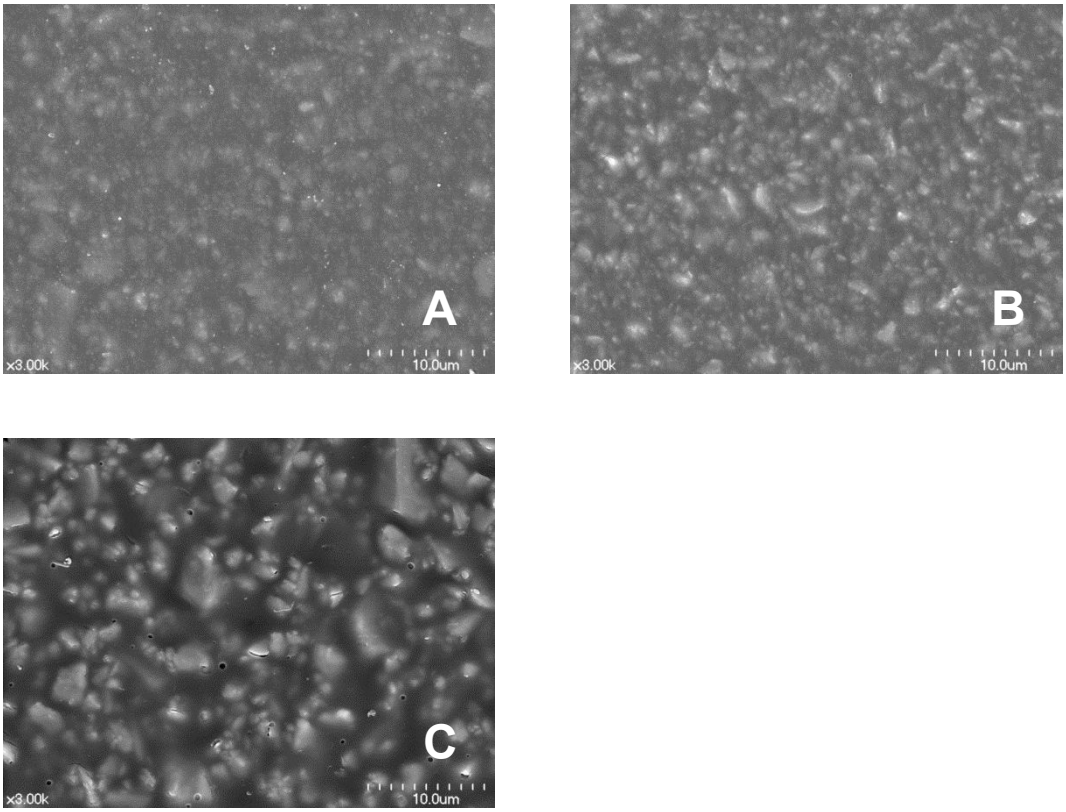

Figure 2. Scanning electron microscopic images of orthodontic adhesives (A) Transbond XT (TX); (B) Transbond Plus (TP); (C) Multi-Cure (MC).

국문 초록

수종의 교정용 접착제의 다세균종 바이오필름 형성 차이 연구

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목적: 본 연구에서는 서로 다른 표면 특성을 가진 수종의 교정용 접착제에 대한 다세균종 바이오 필름의 형성 양상 차이를 평가하였다.

방법: 다음과 같은 세가지 접착제 상에 13종의 세균으로 구성된 다세균종 바이오필름을 형성하였다: composite (Transbond XT, TX), compomer (Transbond Plus, TP), resin-modified glass ionomer (Multi-Cure, MC). 실시간 중합효소 연쇄반응 (real-time polymerase chain reaction)을 이용하여 1일차 (T1)와 4일차 (T2)에 *Streptococcus mutans* (Sm), *Streptococcus sobrinus* (Ss), *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa) 및 전체 세균의 부착량을 측정하였다. 접착제간 세균 조성 차이의 원인을 파악하기 위해 표면 거칠기

(surface roughness, SR), 표면 자유에너지 (surface free energy, SFE) 및 표면 조도 (surface texture)를 분석하였다. 반복측정 분산분석 (repeated measures analysis of variance)을 통해 각 세균종의 양과 전체 세균에 대한 비율의 시간 및 접착제 종류에 따른 차이를 알아보았고, Kruskal-Wallis test를 이용하여 접착제의 SR과 SFE 차이를 확인하였다.

결과: 전체 세균 부착양은 접착제간 유의한 차이가 없었다. 그러나 MC의 경우 다른 접착제에 비해 Sm, Ss 및 Pg가 더 많이 부착하였다. MC는 다른 접착제에 비해 전체 세균에 대한 Sm과 Ss의 비율 또한 높았다. T1에서 T2 사이 Sm, Ss 및 전체 세균 부착양은 증가한 반면 Pg와 Aa는 감소하였다. 그러나 정량분석한 세균의 전체 세균에 대한 비율은 배양 시간이 증가함에 따라 유의하게 감소하였다. MC는 미세한 기공 및 흠으로 인해 TX나 TP보다 더 거친 표면을 보였다. SFE는 TP에서 가장 컸고 다음으로 MC, TX 순이었다. 접착제간 SR 차이는 SFE 차이에 비해 약 10배에 달했다.

결론: 접착제간 SR 차이가 SFE 차이에 비해 더 컸음을 고려해볼 때, MC의 거친 표면으로 인해 다른 접착제에 비해 Sm, Ss 및 Pg가 더 많이 부착된 것으로 보인다.

주요어 : 다세균종 바이오필름, 교정용 접착제, 표면 자유에너지, 표면 거칠기

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