THE RESPONSES OF GINGIVAL AND PERIODONTAL LIGAMENT FIBROBLASTS OT VARIOUS DENTIN CONDITIONED SPECIMENS *IN VITRO*

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I. Introduction

More recently, periodontal therapies have been towards obtaining predictable regeneration of the periodontium. Therefore, the ultimate objective after resolution of the infectious process is regeneration of an organized, functional fibrous attachment.

Required initial events for periodontal regeneration is the attachment and subsequent proliferation of fibroblasts at the root surface. Current evidence suggests that both the gingiva[1,2] and periodontal ligament[3-6] harbor a number of different cells which have a capacity to regenerate the periodontium. In addition, both fibroblasts exhibit clearly different attachment properties *in vitro* in response to various factors[7]. Recent studies suggest that exclusion of gingival tissues, allowing only periodontal ligament cells to attach to root surfaces, favors the formation of a new connective tissue attachment[5,6]. Such difference between fibroblasts of gingiva and periodontal ligament may have important implications in efforts to stimulate regeneration of periodontal tissues and structures following surgical intervention.

The regeneration of fiber attachement to bacterial exposed root surface requires close interaction between periodontal fibroblast and root surface. But, the pathologically exposed root surface may undergo histological, physical, chemical and immunological changes[8]. Many authors have performed studies to establish the best root condition to favor new connective tissue attachment. Modification of root surface by demineralization[9,10], or physical protection of healing sites using membrane[5,11] have been proposed for significant increase in connective tissue attachment.

A variety of agents have been used in conjunction with root demineralization new attachment procedures. Of these, citric acid and tetracycline-HCl have received the most attention. Even if several authors have proposed the use of acid for demineralization, the results have been inconclusive. Therefore, the need for clarification on these points is essential. For these reasons, several *in vitro* and *in vivo* studies have focused on evaluating agents and treatments for their ability to enhance these cells attachment to root surface.

Demineralization has been shown to expose the collagen fibrils of root surface[9,10], which promote chemotactic attraction of fibroblast[12,13]. This could permit the binding of newly synthesized collagen to the collagen of the tooth[9,10].

In order to understand the role of demine-

ralizing surface using citric acid(CA) or tetracyclines-hydrochloride(TC) in connective tissue attachment, GFB and PDL were evaluated for cell attachment and proliferation properties *in vitro*. The degree of fibroblast attachment to the dentin specimens is assessed that the attachment and proliferation of connective tissue cells are necessary for regeneration of new connective tissue attachment.

The objective of this study was to compare the differences in attachment and proliferation properties of GFB and PDL, and the effect of demineralization with either citric acid or tetracycline on cell attachment and proliferation.

II. Materials and Methods

1. Preparation of dentin specimen

Clinically healthy extracted teeth were obtained from oral surgery Department of Dankook University. Following extraction, the teeth were stored in physiologic saline. To prepare the specimens, the roots sliced longitudinally with low speed rotary saw. And, sliced specimen was cut into $3\times3\times0.2mm^3$. Each specimen was square in shape, and grinding on sandpaper(×1000) for flat and smooth surface. The specimens were sterilized to prevent bacterial infection in a graded series of aqueous-ethanol solution(70%, 100 % ethanols) and uv for overnight before dentin conditioning.

2. Cell culture

GFB and PDL were obtained from healthy patients undergoing extractions of premolars for orthodontic reason. For all attachment assays, subcultures between the 2nd and 3rd passages were used.

1) Collection of gingival fibroblasts(GFB)

Sample of gingiva which had been collected before extraction were biopsies of interdental gingival papillae. The sample was washed three times in Hank's balanced salt solution (HBSS, Gibco) with antibiotics. They were dissected into small pieces of abount $0.2mm^3$. The cells were cultured in complete medium ; Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 100 units/ml penicillin(Gibco), 100μg/ml streptomycin(Gibco), and 10% fetal bovine serum (FBS, Gibco) ; at 37℃ in a humidified atmosphere of 5% CO_2-95% air.

2) Collection of periodontal ligament fibroblasts(PDL)

Following extraction from the same patients whom collected GFB, the teeth were washed in HBBS. The crown was dipped in a 5.25% sodium hypochlorite solution form two min to kill bacteria and any remaining gingival cells. And, the teeth were rinsed in three changes of HBSS. Each teeth was placed in a sterile 15ml centrifuge tube with 5ml of 0.05% trypsin and 0.5mM EDTA(Gibco) and 0.1% collagenase(Gibco) and incubated at 37℃ for 90min. After incubation, the tooth was removed, the tube was centrifuged at 20xg for 6 min, and the cells were collected. The pellet was resuspended in complete medium and plated into 60ml culture dishes. The dishes were incubated in a humidified atmosphere of 5% CO_2. The dishes were re-fed daily with complete medium.

3. Dentin specimen conditioning

216 specimens were randomly assigned to each treatment group. Half of the specimens in each treatment group was used in GFB attachment, while another half the specimens was used in PDL attachment. 108 specimens

yield three groups for each treatment, and each 36 specimens yield nine groups for time period(n=4). Three groups of dentin specimens were prepared. And various treatments were applied to the dentin specimens. The first group fo specimens treated with phosphate buffered saline(PBS, control, C) for 5 min. The second group demineralizing in a 24% citric acid solution(CA, pH=1.6), and the third group conditioning with 100mg/ml tetracycline-HCl in deionized distilled water (TC, pH=2.15) was performed by immersion for 5min. After treatment with the acids, all specimens were rinsed several times in sterile PBS. The citric acid concentration selected were based on the Sterrett et al.[14], and the tetracycline-HCl concentration were based on the Terranova et al[15]. The application time were based on the Gregg and Lafferty[16,17]. The solution were continuously stirred for 10min. Both solution were prepared and sterilized by filtration through 0.22μm millipore immediately before using the demineralization.

4. Cell plating

After cell reached confluence, the confluent cell layers were treated with 0.05% trypsin and 0.5mM EDTA for 10min. Cells were centrifuged, and the cell pellets were collected. The collected cells were counted using hemocytometer.

Four specimens in each group were available for each time interval. GFB and PDL were plated into 96 microwell plates, each well of which contained one dentin specimen. Each wells with specimen was prewetted for 1 hr at 37℃ in 100μl of complete medium. To evaluate the effects of cell attachments, cells were plated(6×10^3 cells/well) into each well with complete medium and incubated in humidified atmosphere of 5% CO_2. Following the incubation, for the initial attachment, cells were evaluated over a time course from 0.5 hr to 8 hr(0.5, 1, 2, 4 and 8 hours). To evaluate cell proliferation, cells were observed from 1 day to 7 days(1, 2, 4 and 7 days).

5. Measurement and Analysis

1) Evaluation of initial attachment and proliferation

Dentin specimens were removed at various time from 0.5 hr to 7 days, washed in PBS and stained with toluidine-blue briefly. Each specimen was direct counted in light microscope at magnification of 100 to determine the number of attached cells. The data were expressed as counts per randomly selective field. Effects of attachment were compared within and between the two types of cells. Initial attachment and proliferation was compared between groups of control(PBS treated group), TC demineralized group, CA demineralized group. Also, two types of cells were compared. All counting were run in triplicate.

2) Statistical analysis

Statistical comparisons using the student's *t*-test were made at each time point. Mean values and standard errors are given for each variable in the Tables and Figures. All results were considered to be significant at the 5% critical level. Differences were considered to be significant when *P* values were lower than 0.05.

Ⅲ. Results

1. Initial attachment of gingival fibroblasts(GFB)

No statistical significant differences were found between control and demineralized groups following incubation for various time periods up to 8 hrs(Table 1). By 8 hrs, there was a trend for greater cell attachment to de-

mineralized specimen, but the differences were not statistically significant. There was a tendency for a greater attachment in demineralized group over time compared with the control.

2. Initial cell attachment of periodontal ligament fibroblast(PDL)

Compared to the control specimens, TC resulted in similar attachment and less pronounced changes than those after CA(Table. 2). At 2 days and 8 days, demineralized group had significant increased in cell attachment. PDL had similar effects on initial attachment when compared with GFB. Both the two types of cells, CA was more efficient than TC in the initial cell attachment. No significant differences in initial cell attachment of two types of cells could be observed when they originated from the same patient.

3. Proliferation of GFB

Acid demineralization using either CA or TC enhanced cell proliferation. Especially, TC had greater efficacy(Table 3). In each group, there were rapid increase in proliferation at 7 days. CA group showed greater proliferation by 4 days. However, by 7 days, TC did induce exponential increase in proliferation. On the other hand, CA had no statistically significant difference at 7 days.

4. Proliferation of PDL

Enhanced proliferation in demineralization was similar results as GFB(Table 4). CA causes more extensive changes than TC by 4 days. But, by 7 days, TC group showed greater proliferation

5. Comparison of GFB and PDL

Comparisons were made between the GFB

Table 1. Initial attachment of gingival fibroblasts(GFB)

	0.5 hr	1 hr	2 hr	4 hr	8 hr
C-GF	120.8± 4.7	134.0± 4.8	178.5± 3.7	192.3± 2.1	198.8± 2.9
TC-GF	125.3± 3.7	140.0± 5.0	178.5± 7.0	199.8± 7.9	199.3± 7.9
CA-GF	134.0± 4.2	162.5± 9.1*	183.0± 7.6	201.5± 5.7	212.0± 5.4

mean± SE(*P<0.05)

Data are expressed as the number of attached cell.
C ; control, phosphate buffered saline treated group
TC ; tetracycline demineralized group
CA ; citric acid demineralized group

Table 2. Initial attachment of periodontal ligament fibroblasts(PDL)

	0.5 hr	1 hr	2 hr	4 hr	8 hr
C-GF	130.5± 3.0	145.0± 2.7	165.5± 4.5	192.0± 6.0	232.2± 6.8
TC-GF	128.0± 3.0	140.3± 4.8	182.8± 5.0*	188.3± 8.3	251.8± 5.6*
CA-GF	125.0± 7.0	166.5± 7.5*	202.7± 9.6*	214.5± 8.9	249.3± 7.5*

mean± SE(*P<0.05)

Data are expressed as the number of attached cell.
C ; control, phosphate buffered saline treated group
TC ; tetracycline demineralized group
CA ; citric acid demineralized group

and PDL at each time points. In the initial attachment, although the number of attached cells in two types of cells increased over time, there was no significant differences. There was difference only at 8 hr(Fig 1). In the proliferation, there were different growth rate and significant differences at 8 hr, 2 days, 4 days. GFB proliferation rate differed in PDL, and occured more rapidly.

Table 3. Proliferation of gingival fibroblasts(GFB)

	1 day	2 days	4 days	7 days
C-GF	249.5± 5.9	376.0± 5.9	474.0± 8.3	987.5± 9.7
TC-GF	255.3± 9.4	429.8± 11.2*	492.0± 10.6	2019± 14.5
CA-GF	256.0± 9.6	587.5± 8.1*	601.3± 11.5*	1029± 23.2

mean± SE(*P<0.05)

Data are expressed as the number of attached cell.
C ; control, phosphate buffered saline treated group
TC ; tetracycline demineralized group
CA ; citric acid demineralized group

Table 4. Proliferation of periodontal ligament fibroblasts(PDL)

	1 day	2 days	4 days	7 days
C-GF	269.3± 6.6	282.5± 6.8	371.5± 8.5	946.5± 10.8
TC-GF	281.8± 5.7	312.0± 8.9*	392.0± 9.6	1269± 16.4
CA-GF	299.5± 9.5	351.8± 8.7*	529.7± 13.3*	1005± 27.0

mean± SE(*P<0.05)

Data are expressed as the number of attached cell.
C ; control, phosphate buffered saline treated group
TC ; tetracycline demineralized group
CA ; citric acid demineralized group

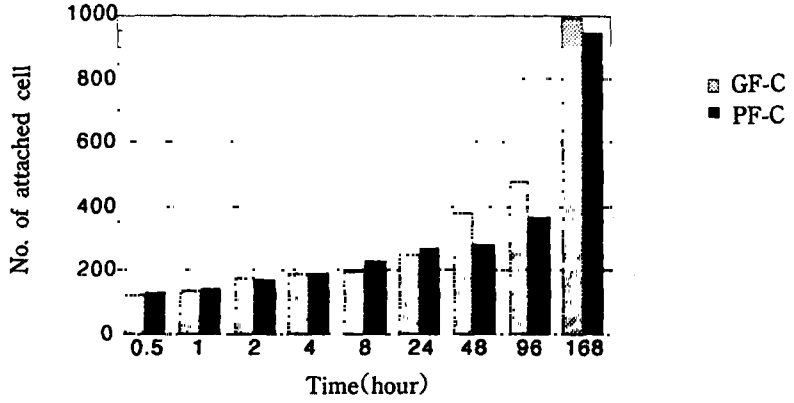

Figure 1. Comparison of gingival and periodontal ligament fibroblasts -control group

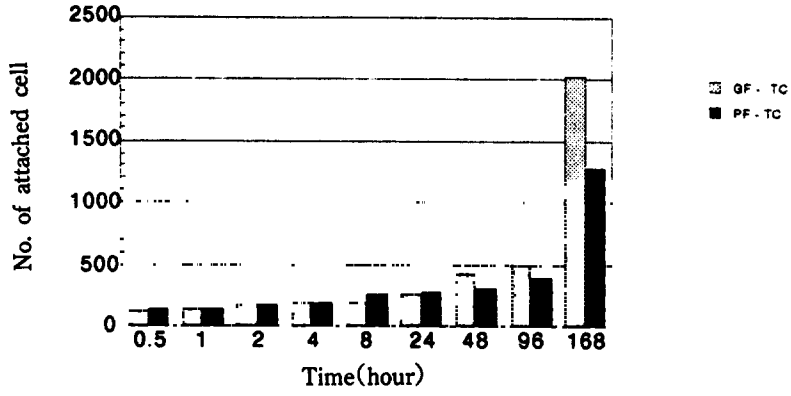

Figure 2. Comparison of gingival and periodontal ligament fibroblasts-TC group

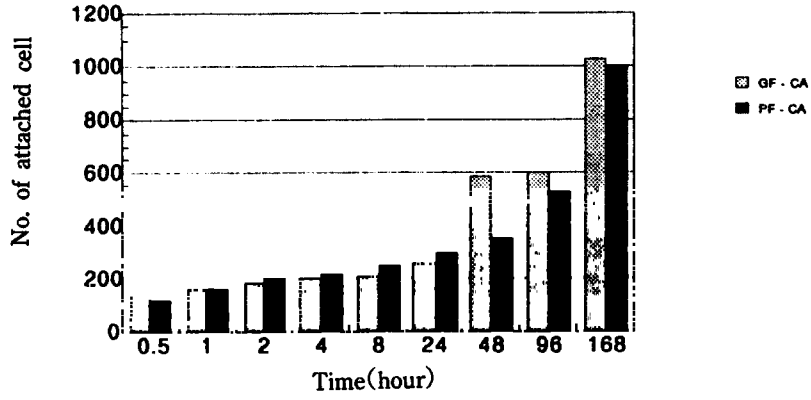

Figure 3. Comparison of gingival and periodontal ligament fibroblasts-CA group

Ⅳ. Discussion

This study was designed for measurement of this initial attachment and proliferation of different types of cells to dentin, and quantifying attachment in response to various dentin conditioning. Any *in vitro* assay which purports to provide insight into the effects of altering the nature of the surface of a root on healing *in vivo*, must be designed to take at least two factors into account : first, initial cell attachment ; and second, prolonged cell proliferation. It is important that initial cell attachment assays include an evaluation of whether or no an agent has an effect on cell proliferation as well. No significant differences in initial cell attachment of two types of cells could be observed when they originated from the same patient. But, the growth characteristics of GFB and PDL exhibit specific differences in proliferative rates. This difference may be attributed to different modes of action of two types of cells in new connective tissue attachment which is one of the major goals in regeneative periodontal therapy.

Acid demineralization of the dentin specimen by CA or TC increased the cell attachment. Cell attachment to dentin is a biologica-

lly active process, and time dependent rate of the reaction. Compared to the control specimens, demineralized specimens had fewer debris and "tidy" appearance(light microscope). A significant amount of debris was noted in control specimens. Attached cell morphology is time dependently similar. Cell attached morphology on demineralized specimen is following as. By 4 hr, the cells were oval shape. At 8 hr, the cells sometimes assumed a spindle shape. Yet, loose and untidy cellular attachment. At 1 day, the cells appeared a polygonal shape with a great number of cytoplasmic extensions. At 7 days, the surface was almost completely covered with fibroblasts. The cells kept their fusiform shape, and looked fairly tidy. These appearance showed that, both the two types of cells, acid demineralization is biocompatible with fibroblastic attachment and proliferation. Our results confirm that dentin conditioning of either CA or TC influenced the initial attachment and proliferation and that could act by different mechanisms. Until 4 days, and both the GFB and PDL, CA demonstrated significantly greater attachment than the corresponding TC. On the other hand, TC favored an enhanced cell proliferation at 7 days. These results showed the importance of the chemical agent utilized to obtain an effective fibroblastic attachment and proliferation. Also, these findings are important to clinical assessment of CA and TC, and indicate that such studies should include the establishment of exact mechanism. Whereas a CA showed a marked effect in initial cell attachment, the proliferation was more persistent by TC. It has been shown to differ markedly depending on whether initial or long term attachment is under investigation. Thus, root conditioningpotential may have significantly augmented by combinding the tetracycline with a citric acid which has more powerful and long term demineralizing effect[13]. However, the results of the present study must be interpreted with caution due to its limited sample size and short observation period.

Demineralization of root surfaces during periodontal therapy has been performed to enhance regeneration of the lost periodontal attachment and a number of agents have been proposed for the demineralization procedure, including CA[19], and TC[20,20].

Clinical studies of Ca have indicated that therapeutic effectiveness are somewhat equivocal[25-27]. Yet, with *in vitro* systems, CA has consistently enhanced features thought to be relevant in the regeneration of periodontal tissues[22-24,28,29]. Although this study showed a greater attachment for CA demineralization of dentin, it had large standard deviations.

Tetracycline has been widely used in the *in vivo* and *in vitro* systems comparable to CA[15,30]. Although TC has a lower demineralizing effect than CA, it has many other advantageous properties. The useful benefits of the TC attributed to their antimicrobial, anticollagenase, and antiinflammatory proerties. Recent studies suggest that it may have a enhancing fibroblast attachment to root surface[7,15]. Althought TC increases cells attachment, the time dependent changes are slower than those produced by CA. It seems that TC is adsorbed to and subsequently slower desorbing rate. Our data differ with the findings of Tsukuda and Gabler, who suggested supposition of testracycline's cytotoxicity and inhibiting of cell adherence result from intercellular[36]. The reson for this discrepancy cannot be explained with certainty.

Demineralization of the root surface has been shown to expose collagen fibrils and creates a zone of demineralized matrix 3~2 μm thick[9,31,32]. The priniciple component of the dentinal matrix is type I collagen[33]. Sterrett

et al.[34] suggested that exposed collagen fibrils are thought to enhance the regenerative potential of the periodontal wound healing by : (1) inducing cementogenesis[35], (2) soft tissue collagen-dentinal collagen splicing[5,11], (3) augmenting fibronectin-fibrin-collagen binding thereby inhibiting epitheial apical migraton[2,10,23], and (4) enhancing fibroblast chemotaxis, migration and attachment[15,30]. Therefore, that the presence of collagen substrate after surface demineralization of the root may have a markedly beneficial influence this aspect of healing. They might influence the rate and quality of wound healing.

Although TC demineralized dentin surface, time-dependent changes were not as pronounced as those observed by CA. Labahn et al.[18] compared dentin morphology after CA and TC conditioning, and concluded that CA caused more extensive changes than TC. This may increase the surface area and amount of exposed collagen avaiable for new attachment.

Attachment of cells to root surface is crucial for regeneration following wound healing *in vivo*, but hypermineralizing changes may have reduced availibity of favorable substrate. In addition, surface contaiminants inhibit growht and viability of fibroblasts[35], and may prevent new connective tissue attachment in initial wound healing response. Therefore, demineralization of root surfaces could help promote wound healing *in vivo*.

But, the ability of acid to demineralize altered surface and provide a substrate conducive for fiber attachment has been questioned. Some investigators have found no beneficial effects of root demineralization in conjunction with periodontal regeneration attempts[5-11], while others have reported this resulted in increased new attachment formation[2,10,23].

In order to determine whether TC or CA have a direct effect on connective tissue attachment, we evaluated the initial cell attachment and proliferation properties of GFB and PDL to demineralizing surface *in vitro*. The results obtained in the present investigation suggest the possibility that altheration of root surfaces by demineralization, or physical barrier membrane of healing sites could enhance the periodontal regenerative potential.

Further studies are underway to confirm the finding of this results and toward clarifying various possibilities, and determining how it is possible to obtain a substrate from periodontitis affected root surface which supports development of a conncetive tissue attachment system.

V. Conclusions

In order to determine whether citric acid or tetracycline have a direct effect on connective tissue cell attachment, we evaluated the initial cell attachment and proliferation properties of GFB and PDL to demineralizing surface *in vitro*.

The results were obtained as follows ;

1. Cell attachment to dentin was a biologically time dependent active process.
2. In each group, there was rapid cell proliferation at seven days.
3. No significant differences in initial cell attachment between GFB and PDL could be observed.
4. In the proliferation, the growth rate of GFB and PDL exhibit specific differences, GFB proliferation rate occured more rapidly.
5. Both the initial attachment and proliferation in relation to the demineralized specimens were significantly greater than undemineralized ones.
6. The citric acid treatment had greater effect

on the initial cell attachment. Whereas, the proliferation was more persistent by tetracycline.

On the basis of these resutls, it is concluded that modification of root surfaces by demineralization could significantly enhance in connective tissue attachment which is one of the major goals in regenerative periodontal therapy.

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다양한 상아질 처리방법에 따른 치은 및 치주인대 섬유아세포의 반응에 관한 시험관내 연구

단국대학교 치과대학 치주과학교실

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치주치료의 궁극적 목표인 치주조직 재생에 대한 관심이 높아지는 가운데 치주조직 재생에 주된 역할을 하는 치주인대세포와 치근면 탈회에 의한 신부착 효과에 관한 많은 연구가 시도되고 있다. 그러나 광범위한 연구에도 불구하고 어떤 치근면 처리가 신부착 형성에 가장 효과적인지는 아직 논란의 대상으로 남아있다. 이에 저자는 결합조직 부착에 의한 재생에 필수적인 치은 및 치주인대 섬유아세포의 부착 및 증식을 tetracycline-HCl(TC)과 citric acid(CA)로 각각 처리된 상아질 표면에서 비교관찰하여 치주조직 재생에의 기여도를 추정하기 위해 본 연구를 시행하였다. 교정치료를 위하여 본원에 내원한 환자의 제일 소구치의 건강한 치은조직을 채취하고, 발치 후 효소처리에 의한 치주인대세포를 얻은 후 각각 세포배양하였다. 상아질 절편은 3×3×0.2mm³로 준비하고 TC과 CA처리군으로 나눈 후 각각 치은 및 치주인대 섬유아세포를 부착시키고 초기부착은 8시간까지, 증식은 7일까지 관찰하여 다음의 결과를 얻었다.

1. 각 군의 세포 부착은 시간이 지남에 따라 증가되었다.
2. 모든 군에서 7일째 빠른 증식상을 보였다.
3. 초기 부착시 치은과 치주인대 섬유아세포의 반응에는 유의한 차이가 없었다.
4. 반면에 치은과 치주인대 섬유아세포는 증식속도에서 차이를 나타내었으며 치은 섬유아세포가 좀 더 빠른 성장을 보였다.
5. 치근면 탈회에 따라 초기부착과 증식상에서 모두 유의성 있는 증가를 보였다.
6. CA는 초기부착에서, TC는 증식상에서 더욱 효과적으로 작용했다.