Influence of the Nature of Dentin Surfaces on Proliferation and Mineralization Activity of Gingival and Periodontal Ligament Cells

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Abstract: The purpose of this study was to investigate the proliferation and mineralization activity of the human gingival and periodontal ligament cells in relation to the nature of the dentin surface. Three types of bovine dentin blocks were used: 1) Rp group: dentin block was root planed by hand scaler, 2) CA group: dentin block was treated by citric acid pH 1 for 3 mins and 3) Na group: citric acid treated block was further exposed to 10% NaOCl for 3 mins. Besides this, hydroxyapatite (HA) blocks were taken as 4th group. Human gingival cells (HGC) and periodontal ligament cells (HPLC) were seeded on the blocks and cultured. Alkaline phosphatase (ALP) activity and protein concentration of the attached cells on blocks were measured at the 1, 3, 5, 7, 10 and 14 days interval. Furthermore, HGC and HPLC were cultured in DMEM and the blocks were kept on the cell sheet after becoming confluent, media was changed to a-MEM supplemented with 100 nM dexamethasone and 10 mM β-glycerophosphate. Mineralized nodules were stained weekly for 3 weeks by von-Kossa method and the area was measured in relation to the margin of blocks.

The results of the protein concentration of the HGC was higher than the HPLC in the 4 groups. Among the 4 groups, protein concentration of HPLC increased at a faster rate in CA group and in HGC, protein concentration increased at a slower rate in Na group. HGC expressed very low ALP activity and was significantly lower than the HPLC. HPLC showed the highest activity in relation to CA group, and lowest activity was observed in relation to Na and HA group. Regarding the mineralized nodule; HPLC formed larger area of nodule than HGC. CA group formed the largest area of nodule in HPLC but there was no difference among the 4 groups in the HGC. The results suggest that the nature of the dentin surface could influence the cellular proliferation and mineralization activity of HPLC, and also the proliferation of HGC. As HGC are able to show mineralization activity in this study though it is very low, it can be suggested that HGC have the potentiality to form calcified tissue.

Key words: Alkaline phosphatase, Proliferation, Dentin nature, Gingival cell, Periodontal ligament cell

要旨: 本研究の目的は、象牙質の表面性状が歯肉細胞と歯根膜細胞の増殖および石灰化能にどのような影響を及ぼすかを検討することである。牛歯から象牙質ブロックを作製し、3群に分けて次の表面処理を行った。1) Rp群：ハンドスケーラーでルートプレーニング、2) CA群：pH1のクエン酸で3分間処理、3) Na群：CA群をさらに10% NaOClで3分間処理。さらにhydroxyapatite ブロックを作製しHA群とし、計4群とした。各ブロック上にヒト歯肉細胞（HGC）、歯根膜細胞（HPLC）を播種、培養した。1, 3, 5, 7, 10, 14日にア
Introduction

Periodontium comprises gingiva, periodontal ligament, root cementum and alveolar bone. Periodontitis causes the destruction of this tissue structure leading to its regeneration a complex phenomenon. Most of the works1, 2) addressed that periodontal ligament cells have the progenitor cells for periodontal regeneration. Guided tissue regeneration was introduced to have a selective cell population in the defect area by excluding cells from the gingival connective tissue and gingival epithelium3–5). On the other hand, gingival fibroblast could not draw so much attention as periodontal ligament cells in this research field. It has been observed that granulation tissue from gingival connective tissue does not have any contribution to the formation of hard tissue essential for regeneration6). But it was suggested that gingival cell under appropriate stimulation may participate in the regeneration of periodontal tissue7). At present the specific roles of periodontal ligament cells and gingival cells, as well as the roles of other cells present in the periodontal tissue, in regulating periodontal regeneration have not been well established8).

Extracellular matrix serves a number of important functions during periodontal wound healing9). It regulates the cellular activity and function. Dentin matrix may be one of the influencing factors in regulating the activity of the healing cells during regeneration. A critical step in periodontal regenerative therapy is to alter the root surface to make it a hospitable substrate to support and encourage migration, attachment, proliferation and proper phenotype expression of periodontal connective tissue progenitor cells10–14). Mechanical and chemical means have been used to promote favorable root surface characteristics. Scaling and root planing are used to remove hard and soft deposits of the cementum or dentin. Several investigators10–13) have reported that removal of the root cementum and demineralization of the denuded dentin by the application of citric acid promotes new connective tissue attachment. Citric acid demineralization exposes the collagen fibrils of the dentin matrix and a subsequent interdigitation of these fibrils takes place with collagen fibrils of the adjacent connective tissue. Organic portion of the dentin is mainly collagen and the inorganic portion consists mainly of hydroxyapatite. The influence of this organic and inorganic substances on the cellular activity can be identified by some biochemical evidence.

Alkaline phosphatase (ALP) is a glycoprotein thought to be involved in the process leading to mineral formation in tissues like bone and cementum15). Recently it was suggested that hard tissue formation activity can be assessed by observing the formation of mineralized nodule in vitro culture system16–20). This activity of the cells can further be evaluated in response to different types of materials.

The purpose of this study was to investigate the influence of the nature of the dentin surfaces, especially the organic and inorganic portion of dentin on the proliferation and mineralization activity of the gingival and periodontal ligament cells (in vitro) by assaying and comparing the ALP activity and protein content of the gingival and periodontal ligament cells which were seeded and cultured on different types of dentin and hydroxyapatite blocks.
In addition to this, a mineralization protocol was maintained in vitro tissue culture to measure and compare the deposition of mineralized nodule in relation to the margin of the dentin and hydroxyapatite by the gingival and periodontal ligament cells.

**Materials and methods**

1. **Cell culture**

   Human periodontal ligament cells (HPLC) were obtained under the patient's concern from a female patient of 13 years old, undergoing extraction of upper left first premolar for orthodontic treatment purpose. The cells were cultured following a modification of the technique described by Kawase et al. Periodontal ligament attached to the middle one third of the root was scraped with a scalpel. The scraped tissues were placed into the 35mm culture dishes and sterile micro cover glasses (Matsunami Micro Cover Glass®, MATSUNAI GLASS IND., LTD., Japan) were given on the tissue explants. The tissues were cultured in DMEM (Dulbecco’s Modified Eagle Medium®, Gibco BRL, USA) supplemented with 10% FBS (Fetal Bovine Serum®, Gibco BRL, USA) and antibiotic (Penicillin 100 units/ml and Streptomycin 100 μg/ml; Gibco BRL, USA). Cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% CO₂. Subcultures within third to sixth passages were used in this study.

   Sample of gingival tissue was voluntarily given by a female patient of 28 years old. Human gingival cells (HGC) were cultured in the same manner as described for the periodontal ligament cells and cells were used between third and sixth passage for all the experiments.

2. **Preparation of the dentin and hydroxyapatite blocks**

   Bovine permanent teeth were collected from the slaughter house immediately after the animal was slaughtered (age, 3 years) and frozen at −80°C until used. After defrosting, the gingiva, periodontal ligament and cementum were removed and 4×3 mm sized 294 dentin blocks of 1 mm thickness were prepared. Dentin blocks were divided into three groups and each group contained equal number of dentin blocks: 1) Rp group, 2) CA group and 3) Na group. Rp group: root planing of the dentin blocks was done by hand curette scaler. CA group: blocks were immersed in the saturated citric acid solution (pH 1) for 3 minutes. Na group: at first the blocks were exposed to saturated citric acid solution (pH 1) for 3 minutes and followed by placing the blocks into 10% NaOCl for 3 minutes. All the blocks were passed through several washes of distilled water and finally, overnight incubation was done in the media composed of DMEM supplemented with antibiotic (Penicillin 1,000 units/ml and Streptomycin 1,000 μg/ml). The blocks were transferred to the same media used for the primary culture.

   In addition to the dentin blocks, 98 hydroxyapatite blocks were used in this experiment. Hydroxyapatite (HA) blocks (Bisoserum®, Bee Brand Medico Dental, Japan) were trimmed to required thickness of 1 mm and the size was about 4×3 mm. The apatite blocks were autoclaved.

3. **Alkaline phosphatase activity**

   HGC and HPLC were seeded separately on the same number of blocks for each group at a concentration of 6.8×10⁴ cells/well in 1.5 ml of the same media used for the primary culture. The ALP activity of the attached cells on the dentin and hydroxyapatite blocks were measured at 1, 3, 5, 7, 10 and 14 days interval using a slightly modified method of Bessey et al. The blocks were washed by sucrose for several times and assays were carried out by immersing the block in a reaction mixture of 1 mM MgCl₂, 25 mM sucrose, 5 mM tris-HCl buffer (pH 10) and 2 mM p-nitrophenyl phosphate (Wako, Japan). All the specimens were incubated for 40 minutes at 37°C. The ALP activity of the reaction products was determined by measuring of absorbance at 420 nm using spectrophotometer (HITACHI U-2000®, Hitachi, Japan) and the data was expressed by dividing with the protein concentration of each block. As a control; 5 mM levamisole (Aldrich chemical company, USA), a specific non-competitive inhibitor of ALP was used in this experiment.

4. **Protein concentration**

   After measuring the ALP activity the blocks were washed several times with sucrose and vibrat-
ed into 10% Sodium Dodecylsulfate (Wako, Japan). Protein content of the attached cells was estimated by the method of Lowry et al.\textsuperscript{23}, using Bio-Rad protein assay kit\textsuperscript{R} (Bio-Rad Laboratories, USA) and bovine serum albumin as a standard. Protein concentration was determined by measuring of absorbance at 750 nm. Protein concentration of each block was divided by it's surface area. Results were expressed as the ratio of protein concentration to the first day.

5. Mineralization protocol

HGC and HPLC were cultured separately in the 35 mm dish at a density of 1 × 10\textsuperscript{5} cells/ml. When the cells became confluent, the media was changed to α-MEM (Alpha-minimum essential media\textsuperscript{R}, Gibco BRL, USA) supplemented with 10% FBS, antibiotic (Penicillin 100 units/ml and Streptomycin 100 \mu g/ml), 100 nM dexamethasone (Wako, Japan) and 10 mM β-glycerophosphate (Wako, Japan). At the same time the dentin and hydroxyapatite blocks were kept on the cell sheet of the dish. To detect the formation of mineral like nodule in relation to the margin of the blocks; the cells along with the blocks were fixed by 95% ethanol and stained weekly for 3 weeks by using von-Kossa method. The specimens were examined under microscope and photographed was taken by Olympus 3 CCD T.V. camera system\textsuperscript{R} (Olympus, Japan) for measuring the area of mineralized nodules attached to the margin of the blocks by NIH image\textsuperscript{R} (Free ware) in the computer (Power Macintosh 7500/100\textsuperscript{R}, Apple Computer Inc., USA). Photograph was taken from the 4 margins of a block and from each photograph, the data was presented by dividing the areas of the mineralized nodules with the length of the dentin and HA margin.

6. Statistical analysis

ALP activity, protein concentration and the area of the mineralized nodules were analyzed with the Mann-Whitney U test using Stat View\textsuperscript{R} (Abacus Concepts Inc., USA) software in the Power Macintosh 7600/200\textsuperscript{R} (Apple Computer Inc., USA).

Results

1. Alkaline phosphatase activity (Table 1)

In the root planed group, ALP activity of the HPLC was low on the 1st and 3rd day and began to increase from the 7th day to 14th day. On the other hand, the HGC did not show any ALP activity on the 1st and 3rd day; and began to express the activity from the 5th day to 14th day. ALP activity of the HPLC was about 7 times higher than HGC on the 14th day.

In the CA group, ALP activity of the HPLC was low on the 1st, 3rd and 5th day; and began to rise from the 7th day and increased rapidly at the 14th day. The gingival cells showed same type of low activity as the Rp group. On the 14th day, HPLC showed about 20 times higher activity than the HGC.

Regarding the Na group, the HPLC showed very

| Table 1 | ALP activity of the HGC and HPLC in relation to the 3 groups of dentin and hydroxyapatite. Values are means±S.D. (\mu mol Pi/min/mg protein). |
|---|---|---|---|---|---|---|
| group | 1 | 3 | 5 | 7 | 10 | 14 |
| HGC Rp | 0±0 | 0±0 | 2.78±4.73 | 11.45±13.89 | 7.86±6.85 | 9.01±4.84 |
| CA | 0±0 | 0±0 | 0.01±0.01 | 3.04±4.47 | 5.01±4.00 | 4.71±2.10 |
| Na | 0±0 | 0±0 | 0±0 | 1.04±2.56 | 5.86±7.26 | 5.25±8.02 |
| HA | 0±0 | 0±0 | 0±0 | 0±0 | 2.64±3.08 | 6.36±6.41 |
| HPLC Rp | 1.39±3.40* | 2.39±3.72 | 0±0 | 10.10±13.54 | 42.58±20.59* | 60.86±9.90* |
| CA | 0±0 | 2.83±3.16 | 3.78±6.19 | 11.01±2.08* | 84.39±17.09* | 110.09±10.73* |
| Na | 0±0 | 2.89±7.09 | 0±0 | 0±0* | 17.18±7.77* | 44.90±13.27* |
| HA | 0±0 | 0±0 | 0±0 | 1.29±3.15* | 6.31±8.56** | 33.59±8.69** |

Significant difference among the 3 groups from root planed group at p<0.05 : *, p<0.01 : **. Significant difference between HGC and HPLC at p<0.05 : †, p<0.01 : ‡‡ (Mann-Whitney U test).
low ALP activity up to the 7th day and from the 10th day it began to increase. ALP activity of the HGC were not detected up to the 7th day and began to increase from the 10th day but the activity was very low as the CA group. On the 14th day, HPLC showed about 8 times higher activity than the HGC.

In the HA groups, ALP activity of the HPLC was not detected on the 1st, 3rd and 5th day, and began to be expressed from the 7th day. HGC presented no ALP activity on the 1st, 3rd, 5th and 7th day; and began to show from the 10th day. HPLC displayed about 5 times higher activity than the HGC on the 14th day.

In the HPLC, CA group showed the highest ALP activity which was about 1.8 times higher than the Rp group, and about 3 times higher than the Na group and HA group. ALP activity of the HGC was very low in the 4 groups and no remarkable difference was observed among all the groups.

2. Protein concentration (Table 2)

In the Rp group, on the 3rd day protein concentration of the HPLC was slightly higher than the HGC but from the 5th day to 14th day HGC became higher than the HPLC.

Regarding the HA group, HGC was higher than the HPLC from the 3rd day to 14th day.

In the HPLC protein concentration increased at a faster rate in the CA group than the other groups. In case of HGC all the groups increased almost in the same manner except Na group where the protein concentration increased at a slower rate than the other groups.

3. Mineralized nodule (Fig. 1, Table 3)

In the Rp group, HPLC formed smaller area of mineralized nodules on the 1st week and on the 2nd week it was characteristically larger than that of the 1st week. In the HGC, the area of the mineralized nodules were smaller than the HPLC from the 1st week to 3rd week. On the 3rd week, mineralized nodule area formed by the HPLC was 2 times larger than HGC on the 3rd week.

In the CA group, mineralized nodule area formed by the HPLC was small on the 1st week and with the passage of time it began to become large which reached to it’s maximum size on the 3rd week. In the HGC, the area of the mineralized nodules were smaller than the HPLC from the 1st week to 3rd week. On the 3rd week, mineralized nodule area formed by the HPLC was 2 times larger than HGC on the 3rd week.

In the HA group mineralized nodule area was very small in relation to the both types of cells on the 1st week. It was increased on the 3rd week but there was no remarkable difference between the HPLC
Fig. 1 Microscopic findings of the von-Kossa stained 3 weeks mineralized nodule area (needle shaped crystallites: arrows) along with the margin of the 3 groups of dentin (D) and hydroxyapatite (HA) blocks in HGC and HPLC (×40).

a: Root planed group in HPLC, b: Citric acid group in HPLC, c: NaOCl group in HPLC, d: Hydroxyapatite group in HPLC, e: Root planed group in HGC, f: Citric acid group in HGC, g: NaOCl group in HGC, h: Hydroxyapatite group in HGC.

and HGC.

Regarding the HA group, both HGC and HPLC formed very small sized nodule on the 1st week. On the 3rd week, both were increased in size but the HPLC formed about 2 times larger area of nodule than HGC.

HPLC in the CA group formed the largest area of nodule followed by the Rp group and the smallest one was formed by the Na group. CA group formed about 2.7 times larger area of nodule than the Na group. Rp group formed 1.7 times larger area of nodule than the Na group. In the HGC, there was no significant variation among the 4 groups throughout the examination period. Mineralized nodules formed by the HGC were smaller than that formed by the HPLC in relation to the 4 groups.
Table 3 Mineralized nodule in relation to the margin of the 3 groups of dentin and hydroxyapatite.

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Discussion

In regenerative attempts, the root surface functions as one of the wound margins and must provide an appropriate environment for cell attachment and differentiation if regeneration is to occur\(^{24,25}\). The influence of dentin substratum on the healing cells may be of even greater importance to periodontal healing at the root surface, where it involves proliferation and differentiation not only to periodontal ligament cells but also other cells involved in the regeneration procedure. Organic portion of the dentin is composed mainly of collagen and non-collagenous protein. On the other hand the inorganic portion of the dentin is composed mainly of hydroxyapatite crystals. In this study, CA group represented the organic portion of the dentin. Inorganic portion was represented by Na group and HA group.

Alkaline phosphatase is a glycoprotein thought to be involved in the process leading to mineral formation in hard tissues like bone and cementum\(^{15}\). Wlodarski et al.\(^{26}\) have demonstrated the ALP as a marker of osteoinductive cells. There is evidence that periodontal ligament cells exhibit characteristics of higher alkaline phosphatase activity than the gingival cells\(^{27,28}\). The data presented in this study concurs with that, i.e. the alkaline phosphatase activity of the periodontal ligament cells in relation to the 4 groups were significantly higher than the gingival cells. In response to different types of materials, HPLC exhibited difference in ALP activity. It was observed that CA group showed the higher ALP activity than the other groups. Partial surface demineralization by citric acid at pH 1 for 3 minutes may influence ALP activity by presenting a favorable microenvironment for the cells, which appears to be important for induction of cementum\(^{29}\). There is inhibition in the ALP activity of the Na and HA group. In case of Na group, it may be due to the disruption of the microenvironment. In case of HA group similar effects were observed in case of pulp fibroblast and osteoblast\(^{30,31}\). ALP activity was measured to assess the cell differentiation and, therefore, increases in the activity of the citric acid treated dentin block in the periodontal ligament cell culture was regarded as an indication of the enhanced differentiation of the periodontal ligament cells into the early stage of mineralized tissue forming cells such as cementoblastic or osteoblastic cells\(^{32}\). In the HGC, ALP activity was not different in response to the various types of dentin and HA blocks. It is not clear why HGC differed from the HPLC in this regard, this may be due to the fact that HPLC and HGC are phenotypically different types of cells\(^{33,34}\).

Cell numbers were determined by measuring the
protein content of the cell layers attached to the dentin and HA blocks. There is evidence that the proliferation of HGC is faster than the HPLC\(^{33,34}\). This is supported by the present finding that the protein content of the HGC was higher than the HPLC in relation to the 4 groups. Among the 4 groups, CA group promoted the cellular growth when compared with the other groups in case of HPLC. The results suggest that citric acid demineralization could contribute to the reported enhancement of healing by improving the attractiveness of the root surface as a substrate to which HPLC can adhere and proliferate\(^{33,34}\). But it is not known why HGC was different in this aspect but it is suggested that HGC were phenotypically different from HPLC\(^{33,34}\).

In this experiment, Melcher's method\(^{35}\) was used with slight modification to determine the mineralized tissue formation activity of the cells attached to the margin of the dentin and HA blocks. Periodontal ligament cells of the rat formed mineralized nodule in vitro when they were treated with dexamethasone, ascorbic acid and \(\beta\)-glycerophosphate\(^{17,19}\). In the present study, it was observed that HPLC formed calcified nodule in relation to the different types of dentin and HA blocks which was about 4 times larger than the nodules formed by the gingival cells. In the HPLC, citric acid treated block influenced the activity most followed by root planed group. This is consistent by Urist and co-workers\(^{36,37,40,41}\), and Huggins\(^{38,39}\), who have demonstrated that demineralized dentin possesses a bone-inductive principle capable of inducing mesenchymal cells to be transformed into osteoblasts, which deposits osseous tissue on the surface of the implanted matrix.

Regarding the HGC, they appear to be capable of producing an osteoid in response to signal received from tooth matrix which is consistent to present finding, where it was shown that gingival cells were able to form mineralized nodule attached to the margin of different types of dentin and HA blocks though the area of nodules was very small in comparison to that formed by periodontal ligament cells and all the blocks in the gingival cell culture showed almost same mineralization activity. In this study, the ALP activity of the HPLC was about 20 times higher than HGC but the area of the mineralized nodule was only 4 times larger than the HGC in relation to the CA group. An explanation for this difference between ALP activity and the area of mineralized nodule is that the present experimental protocol assayed the specific ALP only where as both the specific and non-specific ALP could participate in a process leading to mineral like nodule formation\(^{25}\).

The data of the present experiment suggest that the nature of the dentin surface could influence the ALP activity and the capacity of the HPLC to form mineralized nodule. On the other hand, it is not known why HGC differed from this kind of activity but it may be suggested that under appropriate stimulation by differentiation factors and growth factors, cells from gingiva could theoretically participate in the formation of periodontal tissue i.e. bone, cementum and periodontal ligament\(^{7,42}\). Such an interpretation is consistent with the recent concept of Cho et al.\(^{43}\) who found that the greatest amount of osteoid was formed when gingival connective tissue was allowed to contact with the root surface coated with platelet-derived growth factor. This is also supported by the findings of King et al.\(^{44}\), who have proposed that application of BMP to the root surface after acid conditioning promotes cementogenesis. Therefore, it is interesting to note that in this study HGC were able to express very low mineralization activity which may be further enhanced by the application of growth factors and it will have a great impact on the periodontal regeneration from the clinical point of view in future. The observation and interpretation of the result of this experiment between the gingival and periodontal ligament cells in relation to the different natured dentin and HA block may provide some important guidelines in developing an effective method for stimulating periodontal regeneration.

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