HUMAN CELL(Hum Cell)

Vol. 16 No. 4
Printed in Japan

Original Article

# Effects of Irradiation on Cementum Matrix Cytokins Function during Periodontal Regeneration

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<Abstract> The influence of γ-ray irradiation on a cementum-impregnated gelatine membrane (CGM) was analyzed with emphasis on its function during periodontal regeneration. In brief, proteins were extracted from γ-ray irradiated cementum (γ C). With the γ C protein, sample cells (gingival fibroblasts, periodontal ligament cells, and alveolar bone cells) were co-cultured, and cytological parameters (cell attachment, cell differentiation and alkaline phosphatase activity) were analyzed. Additionally, kinetics of some gene expression was analysed using reverse transcript RT-PCR, which included osteoproteogerin (OPG) / osteoclastogenesis inhibitory factor (OCIF) mRNA. BMP-2 and osteonectin were resistant to γ-rays, and other cytokines involved in regeneration were decreased. Thus, the attachment activity of osteoblasts to γ C protein was higher than that of non-irradiated cementum (control C). The expression of OPG/OCIF mRNA was lower in co-cultured cells with γ C protein than those with in control C protein. Together the results imply that some cytokine in intact cementum prevents the attachment (differentiation) of bone cells onto the root surface, which may explain why the introduction of CGM following gingival flap surgery induces new cementum, new ligament and new bone formation, but CGM irradiated with γ-rays for clinical use causes ankylosis.

**Key words : periodontal tissue regeneration, growth factors, cementum proteins, osteoproteogerin, osteoclastogenesis inhibitory factor**

**[HUMAN CELL 16(4) : 217 - 229, 2003]**

## Introduction

Previous authors have emphasized the usefulness of cytokines such as platelet derived growth factor (PDGF), insulin like growth factor (IGF-1) in periodontal regeneration therapy for the past decade[1),2)]. Recent studies from other laboratories also have discovered a novel type of cytokine in dental cementum which plays an important role as a significant accelerator in connective tissue wound healing. For example, molecular factors stimulating cell attachment were detected from the cementum, which included bone sialoprotein (BSP)[3)], osteopontin[4-6)] and cementum attachment protein (CAP)[7-9)]. Additional examples included CAP[10)], cementum-derived chemotactic factor (CTCF)[11),12)] and cementum "G" extracts[13)] which is a chemoattractant against periodontal tissue cells and cementum-derived growth factor (CGF)[14),15)] as a cell proliferation factor. Interestingly, cementum substances likely can modulate the regenerative process timely and spatially as matricrine, which was suggested from an experiment showing that the formation of new

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cementum, new ligament and new bone is (this must agree with "formation" which is singular) induced when cementum-impregnated gelatine membrane (which contains all kinds of effective proteins) is placed onto the root surface[16),17)].

Periodontal cells (cementoblasts, periodontal ligament cells (PDL), osteoblasts) participate in periodontal tissue regeneration. Although the origin of these cells is not clear, McCulloch et al.[18)] have recently suggested that these precursor cells (osteogenic stem cells) migrate from the vascular channels connecting the endosteal spaces to the periodontal space and differentiate into cementoblasts, PDL cells and osteoblasts, respectively. Cementum-derived proteins are known to play a role in modulation of the chemotaxis and differentiation of precursor cells of each periodontal tissue cell. However, our preliminary study demonstrated that $\gamma$-ray irradiation to the cementum inhibited the integrated formation of new cementum, periodontal ligament and bone, which consequently caused ankylosis. This means that $\gamma$-ray irradiation resulted in the loss of substances that simultaneously orchestrate the formation of new cementum, ligament and bone. The present study was designed to evaluate the change of biological activity in cemental factors after $\gamma$-ray irradiation which is often used for sterilization of biological materials in order to clarify the role of cementum in the process of ideal periodontal tissue healing.

## Materials and Methods

### Source and preparation of cells prior to the experiments

Human gingival fibroblast cells (hGF) were obtained from a volunteer patient (male, 20 years old) during periodontal surgery. The primary culture cells were subcultured 5-9 times in 10% FBS (Invitrogen Corp., Carlsbad, CA, USA) with Dulbecco's modified Eagle medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan).

Human periodontal ligament cells (hPDL) were obtained from the periodontal ligament tissue derived from an extracted healthy tooth of a volunteer patient (male, 12 years old) during orthodontic therapy according to the method of Somerman et al.[19)] The primary culture cells were subcultured 6-12 times in DMEM containing 10% FBS.

Normal human osteoblast cells (hOst) were purchased from CAMBREX Co. (East Rutherford, NJ, USA) and subcultured 4-10 times in a specialized culture medium (osteoblast growth medium; CAMBREX Co.).

Osteoblastic cells were obtained from simian alveolar bone (sAlv) of a monkey (3 years old female). The cells were subcultured 8-14 times in DMEM with 10% FBS.

MC3T3-E1 was purchased from Health Science Research Resources Bank (Osaka, Japan), and subcultured 14-20 times in alpha MEM (Sigma Aldrich Japan Co., Tokyo, Japan ) in 10% FBS.

Mv.1.Lu (CCL64) was purchased from Health Science Research Resources Bank (Osaka, Japan), and subcultured 4-13 times in MEM (Eagle's minimum essential medium; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% FBS.

### Preparation of $\gamma$-ray irradiated bovine cementum particles

Root surfaces of bovine teeth were curetted carefully (approximately 20 strokes) to obtain samples exclusively from superficial cementum. The obtained fragments were subsequently reduced into small particles (namely cementum particles) by means of motor and pestle force. Then cementum particles (control C) were sterilized by Ultraviolet (UV) irradiation for a control study. For the experimental study, the cementum particles ($\gamma$ C) were irradiated with $\gamma$-ray (50 kGy).

### Source of reagents

Enamel Matrix Derivative (EMD) was purchased from Seikagaku Co. (Tokyo, Japan). rh-TGF-$\beta_1$ was purchased from TECHNE Corp. (Minneapolis, MN, USA), and dexamethasone was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### Cell chemotaxis activity

A modified Boyden blind-well chemotactic chamber method was employed[20)]. In brief, cultured cells in log phase proliferation were suspended in FBS free

medium at a density of 1 x $10^5$ cells / ml, and preincubated at 37℃ for 30min. Medium (28 $\mu$ l) with the test samples was applied in the bottom of a Boyden chamber (48-well microchemotaxis chamber: Neuro Probe Inc., Gaithersburg, MD, USA), polyvinylpyrrolidone (PVP) free membrane filter with a pore size of 12 $\mu$ m (Neuro Probe), and the top chamber was positioned for use and preincubated at 37℃ for 15 min. Cell suspension (50 $\mu$ l) was applied to the top chamber and allowed to incubate at 37℃ in a 5% $CO_2$ atmosphere for 3h. Then the filter was placed faced down, and the trapped cells were fixed in methanol and stained with Diff-Quick staining solution, which was checked under a microscope and the number of cells in the chemotaxis were counted.

**Assay for cell adhesion**

To study the effect of $\gamma$ -ray irradiation to cementum particles or other samples on the attachment of the periodontal cells, a non-radioactive assay method proposed by Hou LT[21] was adapted. In brief, a non-tissue culture-treated plastic 96-well plate (Nalge Nunc) was coated with 100 $\mu$ l of DMEM containing 0.5% FBS and the control C, the $\gamma$ C, EMD or Dex for 24 h and blocked with 100 $\mu$ l BSA in FBS free DME for 1h. Log-phase cells were resuspended in DMEM containing 0.5% FBS at a density of 1 x $10^4$ cells / well and plated on a coated plate. After incubation for 225 min at 37℃ (5% $CO_2$), unattached cells were removed by PBS (-), and the culture medium was replaced with 100 $\mu$ l of FBS / phenol red-free DMEM. Then 25 $\mu$ l of MTS solution was added and incubated for another 3 h. To determine the number of attached cells, optical density was measured at 492 nm using a microplate reader (SPECTRAF LOUR : Tecan Co., Ltd., Maennedorf, Switzerland).

**Cell proliferation activity**

The MTS (Promega Co., Madison, WI, USA) assay was adapted[22]. Cultured cells in log proliferation were conditioned with the culture medium plus 10% FBS, and seeded in the 96 well plate at a density of 1 x $10^4$ cells / $cm^2$. After 24 hour incubation, cells were starved of serum for another 24 h. MC3T3-E1 cells were cultured for 3 h with 10 % serum and then starved of serum for 1 h. Cell proliferation was examined by the MTS method after a co-culture period of 3-7 days (the test sample was changed with fresh medium every 3 days).

**Enzyme linked immunosorbent assay**

ELISA was performed to study the kinetics of concentrations of the factors during periodontal regeneration in the presence of cementum particles with or without $\gamma$ -ray irradiation. PDGF-AB (Amersham Biosciences, Corp., Piscataway, NJ, USA), osteonectin (Takara Shuzo Co., Otsu, Japan), TGF-$\beta$ (Morinaga Institute of Biological Science, Yokohama, Japan) and BSP-II (Sangi Co., Ltd., Tokyo, Japan) were measured according to the manufacturer's protocol. In brief, standardized concentrations of growth factors or cementum extracts were added to 96 well ELISA plates coated with monoclonal antibodies (against each antigen) and incubated for 1 h. After washing, biotinylated antibodies were added to each well and allowed to incubate for 1 h. The plates were washed, and peroxidase labeled streptavidin was added to each well, and the wells were incubated for another 1 h. Following washing, *o*-phenylenediamine was added, and absorbance at 490 nm was recorded using an ELISA reader.

**TGF-$\beta$ activity assay**

The presence of bioactive TGF-$\beta$ in $\gamma$ C or control C extracts were determined by using a specific bioassay, which is based on the ability of TGF-$\beta$ to inhibit proliferation of the mink lung epithelial cell line (known as the Mv.1.Lu cell)[23,24]. Mv.1.Lu cells in log-phase (in 10 % FBS-MEM) were seeded to the 96 well microplate (Nalge Nunc) at a concentration of 1 x $10^4$ cells / well. After an initial 3 hours of culture allowing cells to attach, cells were then starved of FBS for 1 h. Cementum extracts or standardized concentrations of rhTGF-$\beta_1$ (Techne) were then added and cultured for an additional 48 h at 37℃ (5% $CO_2$). At the end of the culture, viable cells were counted by a fluorometric indicator method. The media was changed to phenol red and serum free MEM supplemented with 10 % alamar Blue (Wako pure chemical industries, Osaka, Japan). Followed by 6 hour incubation, the

fluorescence was measured under the following conditions; an excitation wave length of 550 nm and an emission wave length of 590 nm. By comparison with the standard curve, the concentration of total and biologically active TGF-$\beta$ present in the cementum extract was calculated.

**Alkaline phosphatase (ALP) activity assay**

ALP activity was determined as follows: cells were seeded in 35 mm dishes, and cultured with 10% FBS containing medium until reaching confluence. Followed by serum starvation for 48 h, the culturing was continued for another 40 days, and the medium was changed for the test sample every 3 to 7 days. The cultured cells were then harvested and ultrasonicated with 200$\mu$l of PBS (-). Followed by sonication, the cells were centrifuged for 10 min at 15,000 rpm at 4℃, and the supernant was used as a crude enzyme. To 20$\mu$ l of the crude enzyme, 0.02 M of *p*-nitrophenylphosphate and 0.01 M magunecium chloride in 80$\mu$l of Tris-HCl (0.1 M, pH 8.8) was added as a substrate and incubated at 37℃. The extinction was read using a microplate reader (a wave length of 405 nm) after adding the substrate and subsequent incubation (1 to 120 min). One unit of enzyme activity was defined as 1$\mu$M p-nitrophenol released per minute (1U=66.6 $OD_{405}$/min; data not shown). Enzyme activity was presented per mg-protein by measuring the whole protein concentration in the supernatant by a method of Bradford[25].

**RT-PCR analysis of mRNA of TGF-$\beta$, OCN, BMP-2, Osteoproteogerin/ osteoclastogenesis inhibitory factor**

Cells were seeded in a 60 mm dish and cultured in the medium with 10% FBS until it reached confluence. Followed by the serum starvation for 24 h, total RNA was isolated using a SV Total RNA Isolation Kit (Promega). RT-PCR was performed adapting a TaqMan One-Step RT-PCR method. Primers and TaqMan Probe were designed based on the published sequence data of OCN, BMP-2A, OPG / OCIF as shown in Table 1. For TGF-$\beta$ and 18S rRNA, pre-developed TaqMan Assay Reagent, TaqMan rRNA, and control reagents (Applied Biosystems, Foster, CA, USA) were used, respectively. 5-17 ng of total RNA was processed for reaction according to the manufacturer's protocol, and subjected to RT reaction at 48℃ for 30 min. After polymerase activation at 95℃ for 10 min, the annealing and extension were performed at 60℃ for 60 cycles. An increase of fluorescence of the reporter dye was measured on a real time base . PCR products were subjected to SDS-PAGE to confirm the presence of targets. The data were subjected to analysis by means of GeneAmp 5700 SDS (Applied Biosystems) or a Smart Cycler System (Takara Shuzo Co., Otsu, Japan) .

**Table 1:** Sequence of primers and TaqMan probe

| Target | | Sequence (5'-3') |
|---|---|---|
| OCN | Forward primer | GGC TCC CAG CCA TTG ATA CA |
| | Reverse primer | CAG AGT CCA GCA AAG GTG CA |
| | TaqMan probe | CTG CCC TCC TGC TTG GAC ACA AAG G |
| BMP-2A | Forward primer | CCA TTG TTC AGA CGT TGG TCA A |
| | Reverse primer | CAG CAT CGA GAT AGC ACT GAG TTC |
| | TaqMan probe | CTA AGA TTC CTA CGG CAT GCT GTG TCC CGA |
| OPG/OCIF | Forward primer | ACC TTG AGA TAG AGT TCT GCT TGA AAC |
| | Reverse primer | CCA TCT GGA CAT CTT TTG CAA AC |
| | TaqMan probe | TGC AAG CTG GAA CCC CAG AGC G |

## Results

### Physical properties change by γ-ray irradiation

The *in vitro* experiment showed that a 30 minute treatment in PBS (-) results in complete extraction of the substance involved in the chemotactic activity and adhesion activity from the cementum. The quantity of protein extracted from the cementum after γ-ray irradiation was 1.052 mg / 300 mg, and the quantity from the cementum after UV irradiation was 0.792 mg / 300 mg (by the method of Bradford), which demonstrated that the γ-ray irradiation resulted in more extraction of protein during PBS (-) treatment. The extraction pattern showed that the molecular weight of protein resulting from after gel filtration chromatography tends to be lower (Fig. 1).

### Change of the main bone matrix and cytokine quantity in cementum

After γ-ray irradiation, the amount of PDGF, IGF-1 and TGF-β in the cementum decreased, but BSP-II and osteonectin showed no meaningful change (ELISA value). Especially TGF-β activity against the Mv.I.Lu cell proliferation hindrance decreased by 82% (1.82μg /ml in the control C group, 0.32μg / ml in γ C group) as shown in Table 2.

### Change of the biological activity by γ-ray irradiation on cell chemotaxis Cell chemotactic activity

Nishimura et al.[11] showed that cementum has bioactive substances, which chemoattract undifferentiated mesenchymal stem cells in the periodontal space to the root surface, and the stem cells differentiate to the cementoblasts *in vivo*. This indicates that chemotaxisis of the stem cell to the root surface is essential in periodontal regeneration as a pre-step towards cell adhesion. This chemotactic activity was observed in the present *in vitro* experiment as well. The chemotactic activity of hGF and hPDL cells by the PBS extracts from the γ C was considerably less than that from the UV irradiated cementum particles. No decrease of chemotactic activity, however, was observed in the experiment with sAlv cells (Fig. 2).

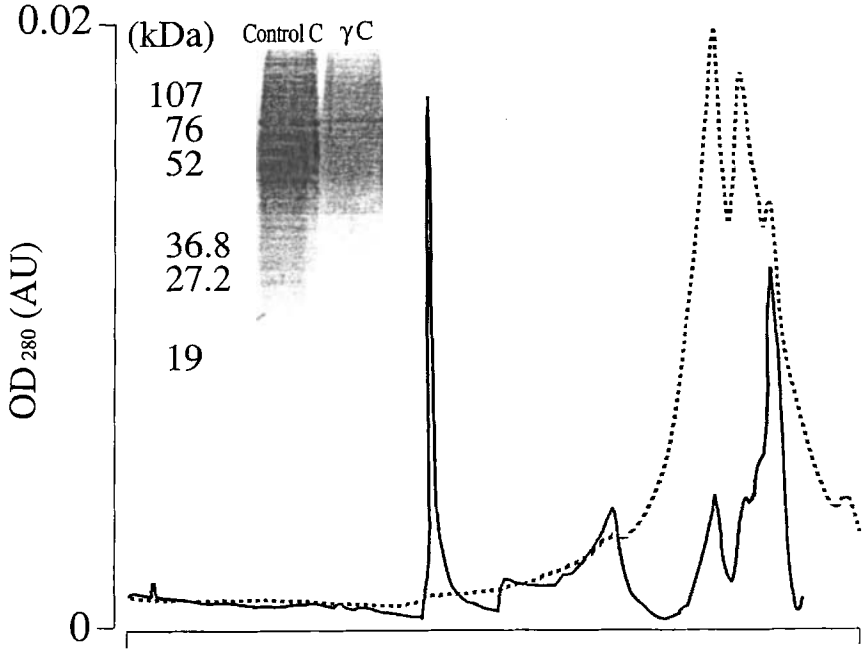


**Fig. 1:** The main band of the cementum extracts, 67 kDa band in SDS-PAGE, disappeard after γ irradiation. gel filtration chromatography chart (solid line: unirradiated protein, dotted line; γ-ray irradiated protein) shows that the protein became small in molecular weight as the result of protein degradation.

**Cell adhesion activity**

Cementum has bone matrix substances with cell adhesion activity such as BSP and CAP as well as collagen (a major component of the cementum). The changes in the cell adhesion activity after $\gamma$ -ray irradiation are shown in Fig.3. The number of attached cells to $\gamma$ C was greater ($p>0.1$) than that of control C in the osteoblastic cell line, sAlv (*) and MC3T3-E1 (**). This suggests that adherent bone matrix of BSP or collagen was not affected by $\gamma$ -ray irradiation.

**Cell proliferation activity**

After $\gamma$ -ray irradiation, cementum decreased its activity to proliferate hPDL cells, but no changes in effects toward hGF or sAlv cells were shown (Fig 4).

**ALP activity**

In the experiment of all the specimens including hGF, hPDL and sAlv, ALP activity was higher in $\gamma$ C, which is in good agreement with the results in the *in vivo* experiment of bone formation stimulation (data not shown).

**The expression level of osteogenesis related gene**

There are two possible causes that may explain how ankylosis occurs when the $\gamma$ C is applied. One is a direct reason; growth inhibitory factors for osteoblasts in cementum are inactivated by $\gamma$ -ray irradiation. The other is indirect; the growth of osteoblasts is amplified due to the loss of a bone formation inhibitory factor secreted from periodontal tissue cells. Therefore, the expression levels of osteogenesis related to gene in periodontal tissue cells was assessed by means of RT-PCR.

BMP-2A, osteocalcin and the TGF-$\beta_1$ expression level of hGF in the co-culture with $\gamma$ C were much lower than that in control C (Fig 5). On the other hand, the expression level of OPG /OCIF in the $\gamma$ C group was lower in hGF and higher in hOst cells.

Induction was performed to hGF, hPDL and hOst cells for 12 hours. This expression decreased in hGF cells after $\gamma$ -ray irradiation, but increased in hOst cells after $\gamma$ -ray irradiation, which resulted in the extended bone formation (Fig 6).

**Table 2:** The concentration of cytokine in cementum extracts. The cementum seems to have enough amount of various substances that induce periodontal tissue regeneration. One of them, TGF-$\beta$, is supposed to be most profoundly influenced.

| Analysis | Control C | $\gamma$C |
|---|---|---|
| PDGF-AB* | 58 pg/ml | 45 pg/ml |
| IGF-I* | 107 ng/ml | 98 ng/ml |
| Osteonectin* | 45 ng/ml | 57 ng/ml |
| BSP-II* | 70 ng/ml | 85 ng/ml |
| TGF-$\beta_1$** | 1.82 μg/ml | 0.32 μg/ml |

* Tested by ELISA.

** Tested by bioassay.

## Discussion

For periodontal regeneration, most important factor is the formation of the periodontal ligament between the two hard tissues (surface cementum and alveolar bone) that acts as a shock absorber during mastication. In idealistic periodontal regeneration, osteoblasts differentiation and proliferation to the root surface are inhibited. Only cementoblasts differentiate and proliferate on the root surfaces, which result in cementum formation and periodontal ligament formation. Additionally, the bone formation by osteoblasts occurs around the destructed bone at the opposite side. In other words, each cell is required to integrate such hard and soft tissues into this restricted area. Our *in vivo* study[16),17)] has already shown that the cementum component regulates this complex regenerative process in terms of time and space. Interestingly, the cementum substances, which had received $\gamma$ -irradiation for sterilization, resulted in only alveolar bone regeneration, and direct adhesion occurred between the newly formed bone and the root surface.

In the process of tissue regeneration where cementum-impregnated gelatin membrane was adapted, the cementum substances resulted in 1) chemotaxis of undifferentiated mesenchymal fibroblasts towards the root surface and 2) differentiation exclusively to cementoblasts[17)]. This means that the cementum substances have a direct function of inhibiting chemotaxisis of osteoblasts towards the vicinity of the root and subsequent proliferation.

Based on such results, the present experiment examined the cell chemotaxis activity and proliferation inhibition activity towards osteoblasts in the cementum. The chemotaxisis activity was decreased substantially after $\gamma$-ray irradiation, and as for proliferation activity no difference was observed between the $\gamma$-ray un-irradiated group and the irradiated group. However, the substance with the

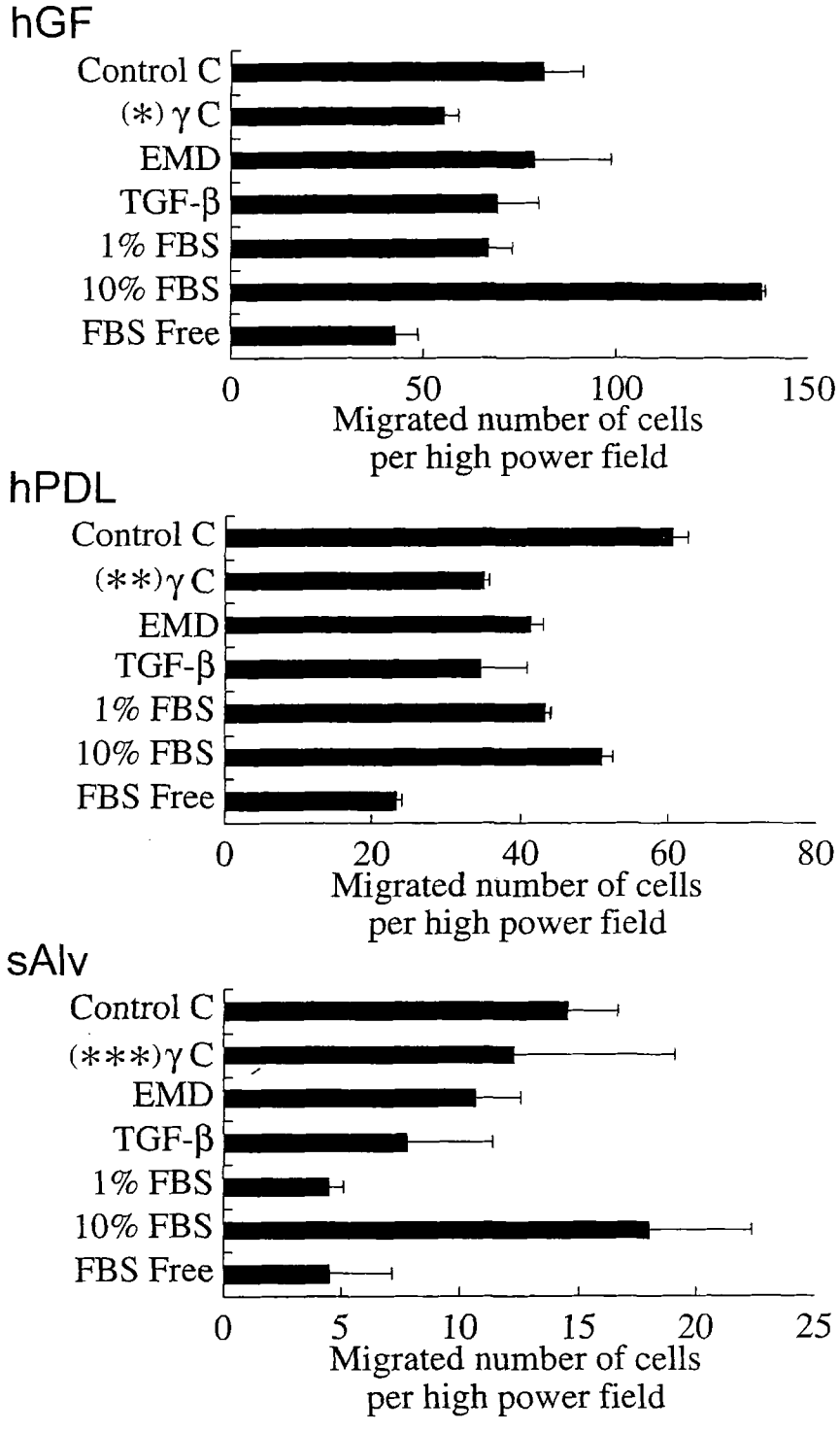


**Fig. 2:** Cell chemotactic activity in the cementum was measured by means of the Boyden chamber. There was chemotactic activity of hGF (*) and PDL (**) cells towards cementum, which was decreased after $\gamma$-ray irradiation but this tendency was not observed as to sAlv cells (***).

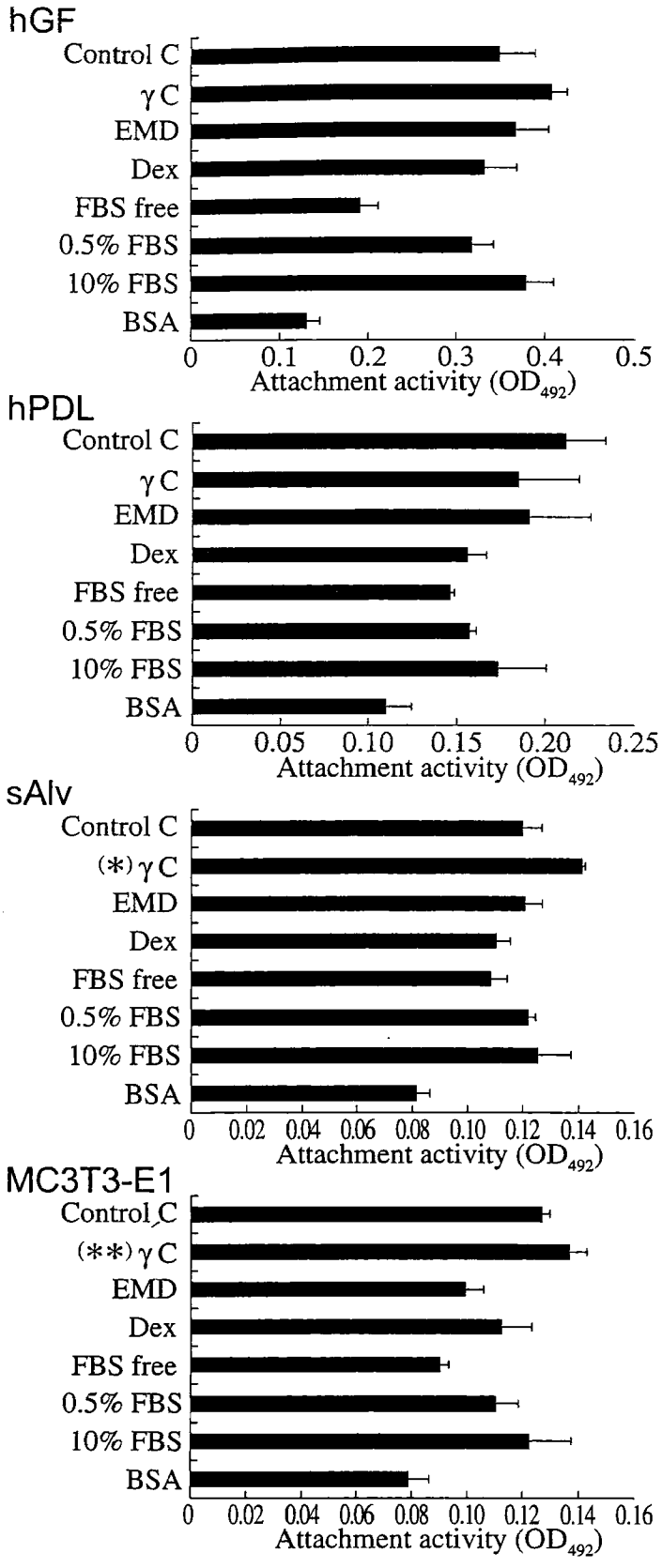


**Fig. 3:** Cell adhesion activity by the cementum extracts. This activity was higher in the $\gamma$ -ray irradiation group than in the inirradiated group except for the case where hPDL cells were used. This suggests that one of substances with cell adhesion activity (collagen, BSP, osteonectin) was not influenced (such as degradation).

adhesion activity of the cell is tolerant to the γ-ray, and the activity increased, which means that the γ-ray irradiation to the cementum results in contribution to bone formation by offering more space.

On the other hand, as an indirect influence, there is a possibility that various cells in the periodontal space may cause inhibition of bone formation in response to stimulation of cementum substance. Recent reports[26), 27)] showed that human dental mesenchymal cells express OPG / OCIF mRNA, and that human periodontal ligament cells secrete OPG / OCIF, one of which report[27)] proposed the hypothesis that OPG / OCIF secreted by the PDL cells in the periodontal space inhibits osteoclasts activation during inflammation. We, however, hypothesized that OCIF may also account for why osteoblasts do not appear at the root surface during periodontal regeneration which is probably due to the existence of root cementum.

Therefore, the expression level of OPG / OCIF mRNA was checked in cells including hGF, hPDL and

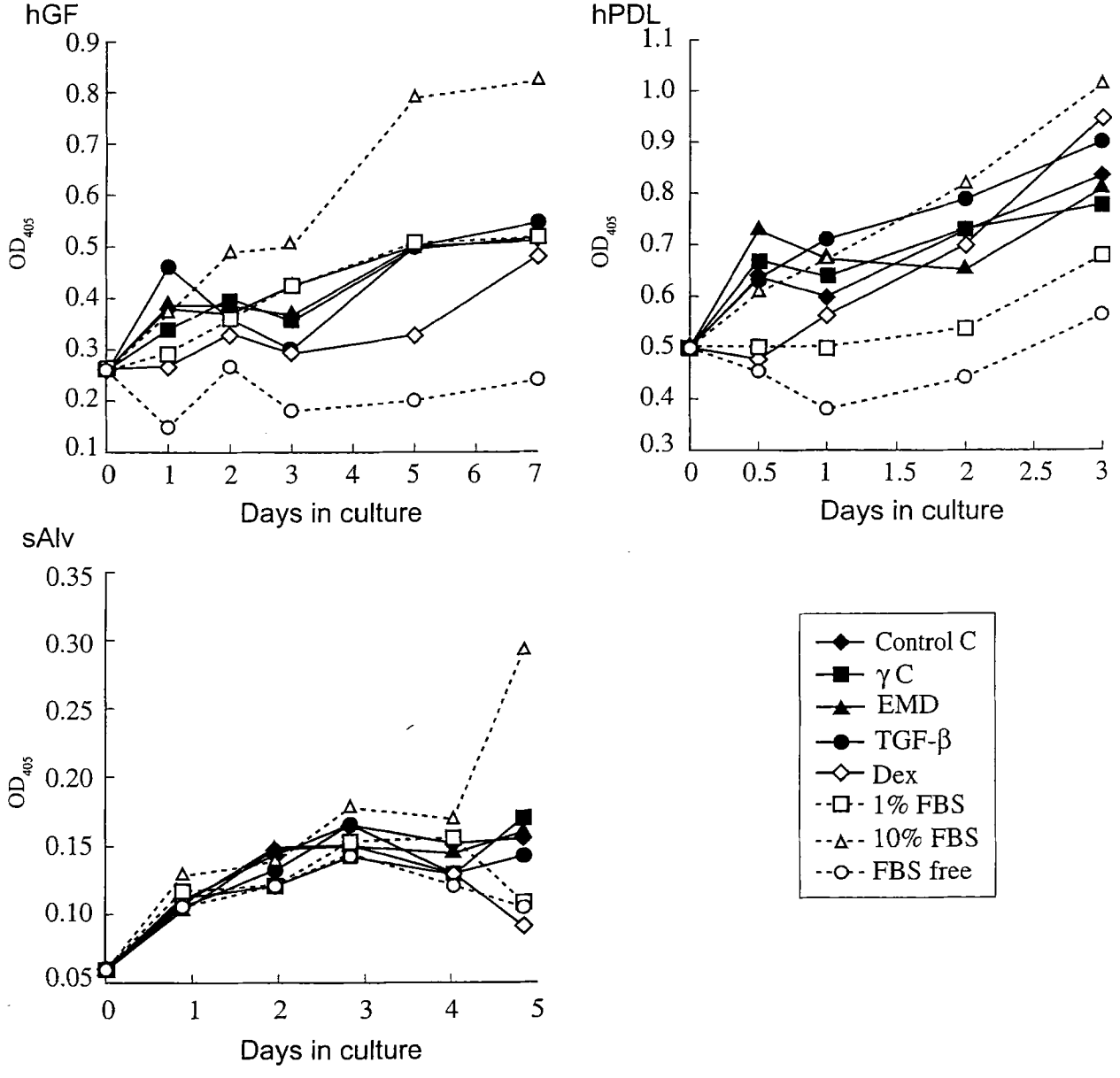


**Fig. 4:** After γ-ray irradiation, cementum decreased its activity to prolife hPDL cells, but such decrease was not observed when hGF and sALv cells were used.

hOst. The result showed that PDL cells expressed the same mRNA level of OPG/OCIF regardless of the γ-ray irradiation dose, but the hGF cell expressed an apparently lower level after γ-ray irradiation. It seems to be necessary to examine osteoclast activity to clarify whether or not OPG / OCIF is secreted in an active form.

Sakata et al.[26] showed that hGF cells express a higher level of mRNA of OPG /OCIF from hGF cells, which contribute to inhibition of bone formation. In their experiment, the expression level of OPG / OCIF mRNA in PDL cells increased by 190% after IL-IB administration, and by 110% after TNF-β administration, but they did not increase after TNF-β and IL-6 administration. However, IL-La and TNF-β were not detected in the cementum which was used for the experiment, which suggests the presence of another kind of γ-ray sensitive factor in the cementum.

In conclusion, the substances that enhance OPG / OCIF secretion of hGF cells in γ C are destroyed by γ-ray exposure, and consequently the proliferation of osteoblasts can not be controlled, and the adhesion place is generated. These events may likely result in bone adhesion.

**Ackowledgment**

This work was supported by Grant-in-Aid for

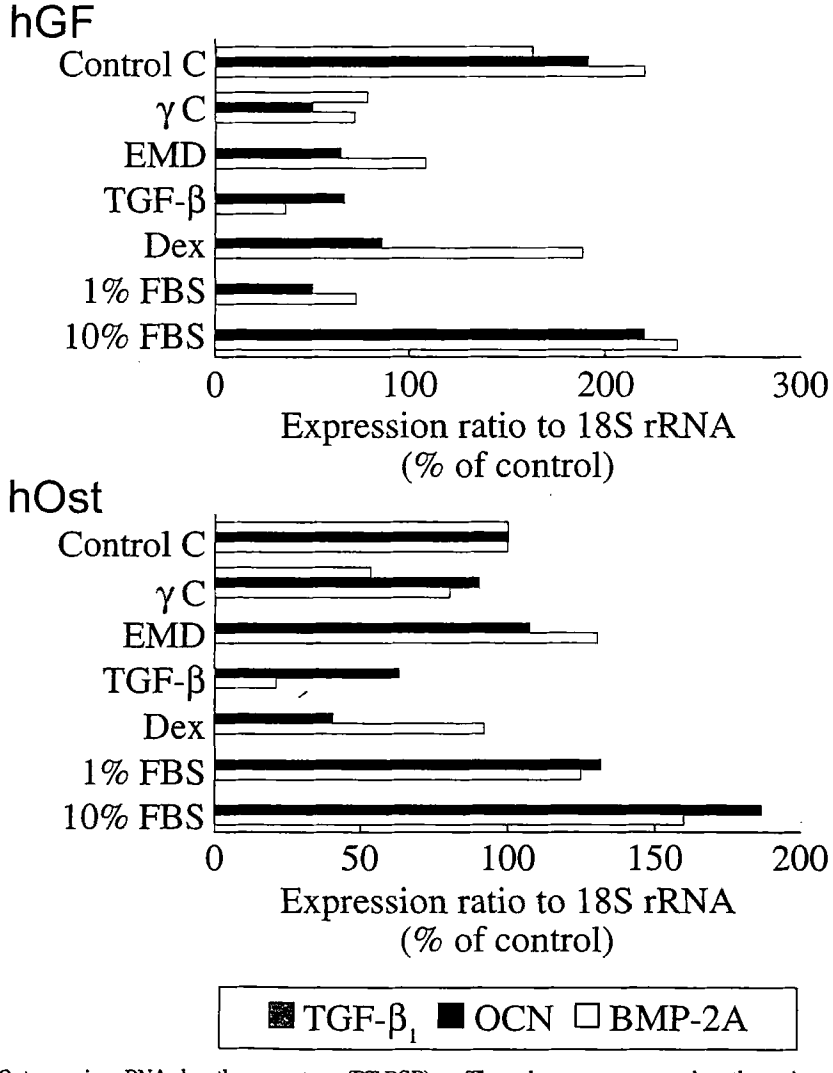


**Fig. 5:** Induction of Osteogenic mRNA by the ementum (RT-PCR). The value was expressed as the ratio against that of 18S rRNA. Induction was performed to HGF cells for 5 days, the both cells induced osteogenic mRNA expression. This expression was inhibited after γ-ray irradiation, which was prominent when hGF cell were used.

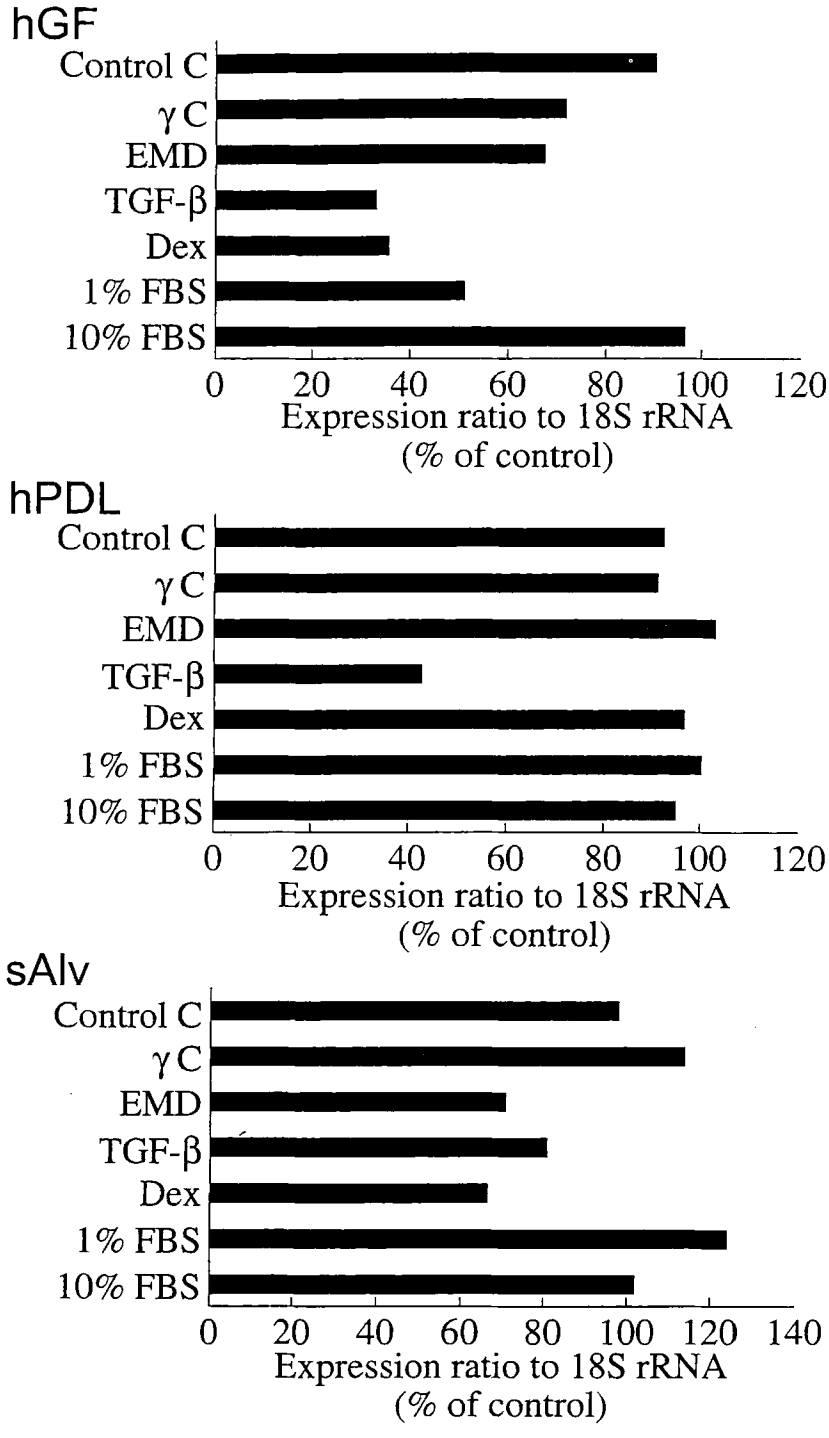


**Fig. 6:** Induction of OPG/OCIF mRNA by the cementum (RT-PCR). The value was expressed as the ratio against that of 18S rRNA. Induction was performed to hGF, hPDL and hOst cells for 12 hours. This expression decreased in hGF cells after $\gamma$-ray irradiation, but increased in hOst cells after $\gamma$-ray irradiation. This suggested that control C usually stimulates hGF cells to secrete OPG/OCIF to preserve the periodontal space, but OPG/OCIF secretion was inactivated by $\gamma$-ray irradiation, which resulted in the extended bone formation.

Scientific Research (KAKENHI) from the Ministry of Education, Science and Culture in Japan (No.10470410 and 1420899)

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Received 2003. 12. 9, Accepted 2004. 1. 20

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