Inhibitory effects of green tea polyphenol (-)-epigallocatechin gallate (EGCG) on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts

Jeong-Ho Yun

The Graduate School
Yonsei University
Department of Dental Science

Inhibitory effects of green tea polyphenol (-)-epigallocatechin gallate (EGCG) on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts

A Dissertation Thesis
Submitted to the Department of Dental Science
and the Graduate School of Yonsei University
in partial fulfillment of the
requirements for the degree of
Master of Dental Science

Jeong-Ho Yun

June 2004

This certifies that the dissertation thesis of Jeong-Ho Yun is approved.

Thesis Supervisor: Seong-Ho Choi

Yun-Jung Yoo

Chang-Sung Kim

The Graduate School

Yonsei University

June 2004

감사의 글

이 작은 결실을 맺을 수 있도록 부족한 저를 항상 따뜻한 관심과 지도로 격려해 주시고 이끌어 주신 최성호 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 격려를 해주신 김종관 교수님, 채중규 교수님, 조규성 교수님, 유윤정 교수님께 진심으로 감사드립니다. 또한, 본 연구에 많은 관심과 도움을 주신 김창성 교수님께도 감사의 마음을 전합니다.

본 연구 내내 많은 도움을 아끼지 않은 백정원 선생님과 치주과 의국원 여러분께 고마움을 전합니다.

항상 곁에서 든든하게 후원해주시고, 언제나 끝이 없는 사랑으로 저를 감싸주시는 아버지, 어머니, 장인, 장모님께 감사드립니다.

마지막으로, 늘 아낌 없는 사랑으로 나를 복돋아 주고 헌신적인 도움으로 따뜻한 버팀목이 되어준 사랑하는 나의 아내 지현이에게 다시 한번 감사하며 진정으로 고마움을 담아 이 논문을 드립니다.

모든 분께 진심으로 감사 드립니다.

2004년 6월

저자 씀

Table of Contents

Abstract(English) vi

Ⅰ. Introduction 1

Ⅱ. Material and Methods 4

A. *P.gingivalis* culture and preparation of sonicated *P.gingivalis* extracts (SPEs) 4

B. Preparation of primary osteoblastic (POB) cells and bone marrow cell 5

C. Cytotoxicity assay 6

D. Reverse transcription-polymerase chain reaction (RT-PCR) 7

E. *In vitro* formation of osteoclasts 9

F. Statistical analysis 9

Ⅲ. Results 10

A. Effect of EGCG on cell viability 10

B. Effect of SPEs and EGCG on the expression of MMP-2, -9, and -13 mRNA in POB cells 11

C. Inhibitory effect of EGCG on osteoclast formation 13

Ⅳ. Discussion 15

Ⅴ. Conclusion 19

References 21

Abstract(Korean) 28

List of Tables and Figures

Table 1. Synthetic oligonucleotide primer used for RT-PCR 8

Figure 1. The effect of EGCG on the viability of POB cells and co-culture 10

Figure 2. The effect of SPEs and EGCG on the expression of MMP-2, -9, and -13 mRNA 11

Figure 3. The effect of EGCG on osteoclast formation 13

Abstract

Inhibitory effects of green tea polyphenol (-)-epigallocatechin gallate (EGCG) on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts

Alveolar bone resorption is a characteristic feature of periodontal diseases and involves the removal of both the mineral and organic constituents of the bone matrix, which is caused by either multinucleated osteoclast cells or matrix metalloproteinases (MMPs). The gram-negative bacterium, *Porphyromonas gingivalis* has been reported to stimulate the activity and expression of several groups of MMPs, whereas (-)-epigallocatechin gallate (EGCG), the main constituent of green tea polyphenols, has been reported to have inhibitory effects on the activity and expression of MMPs.

In the present study, we investigated the effects of the green tea polyphenol, EGCG, on the gene expression of osteoblast-derived MMP-2, -9, and -13, stimulated by *P.gingivalis*, and on the formation of osteoclasts. The effect of EGCG on the gene expression of MMPs was examined by treating mouse calvarial primary osteoblastic (POB) cells with EGCG (20 μM) in the presence of sonicated *P.gingivalis* extracts (SPEs). The transcription levels of MMP-2, -9, and -13 were assessed by reverse transcription-polymerase chain reaction (RT-PCR). The effect of EGCG on osteoclast formation was confirmed by tartrate-resistant acid phosphatase (TRAP) staining in a co-culture system of mouse bone marrow cells and calvarial POB cells.

Treatment with the SPEs stimulated the expression of MMP-9 mRNA and

this effect was significantly reduced by EGCG, whereas the transcription levels of MMP-2 and MMP-13 were not affected by either the SPEs or EGCG. In addition, EGCG significantly inhibited osteoclast formation in the co-culture system at a concentration of 20 μM.

These findings suggest that EGCG may prevent the alveolar bone resorption which occurs in periodontal diseases by inhibiting the expression of MMP-9 in osteoblasts and the formation of osteoclasts.

Key words : matrix metalloproteinase; (-)-epigallocatechin gallate; osteoblast; osteoclast

Inhibitory effects of green tea polyphenol (-)-epigallocatechin gallate (EGCG) on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts

Jeong-Ho Yun, D.D.S.
Department of Dental Science, Graduate School,
Yonsei University
(Directed by Prof. Seong-Ho Choi, D.D.S., M.S.D., PhD.)

I. Introduction

Alveolar bone resorption is the clinically most important issue in human periodontitis, because it leads to tooth loss (Schwartz Z et al. 1997). Bone resorption involves the removal of both the mineral and organic constituents of the bone matrix. Osteoclasts, the cells principally responsible for this process (Baron R 1989), acidify the sub-osteoclastic resorption zone leading to the dissolution of minerals (Blair et al. 1989), while the organic matrix (mainly type-Ⅰ collagen) is degraded by proteolytic enzymes. Osteoblastic cells play a role in the initiation of bone resorption by releasing matrix metalloproteinases (MMPs) that degrade the non-mineralized osteoid layer (principally type-Ⅰ collagen) covering the bone surface, thereby exposing the underlying

mineralized matrix to osteoclastic action (Chambers et al. 1985a; Chambers et al. 1985b; Delaisse et al. 2000).

MMPs are a family of zinc-dependent endopeptidases, including collagenases, gelatinases and stromelysins, which have the combined ability to degrade the organic components of connective-tissue matrices (Birkedal-Hansen 1993; Reynolds et al. 1994; Reynolds et al. 1997). Not only collagenase (MMP-1 and MMP-13), but also gelatinase A (MMP-2) and B (MMP-9), have been considered the principal MMPs in the digestion of bone collagen by osteoblasts (Heath et al. 1984; Meikle et al. 1992; Hill et al. 1995; Kusano et al. 1998; Mizutani et al. 2001). It was reported that while collagenase is responsible for the initial cleavage of native type-Ⅰcollagen, gelatinase A and B may play a significant role in the subsequent digestion of the denatured collagen fibrils (Hill et al. 1995).

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, is implicated as an etiologic agent in periodontal diseases (Moore WEC et al. 1987; Slots et al. 1988; Dzink et al. 1988). Even though periodontal destruction is partly caused by proteinases secreted from this group of bacteria, it is now accepted that the host response to such bacterial products is the major cause of the pathogenesis (Birkedal-Hansen 1993). Furthermore, the products of *P.gingivalis*, such as lipopolysaccharide (LPS), membrane proteins and bacterial proteinases have been shown to have the ability to induce and activate host MMPs (Uitto et al. 1989; Sorsa et al. 1992; Fravalo et al. 1996; DeCarlo et al. 1997; Chang et al. 2002; Grayson et al. 2003; Pattamapun et al. 2003).

Green tea is one of the most popular beverages in the world, and it has received considerable attention because of its many scientifically proven beneficial effects on human health. Several epidemiologic and experimental observations have confirmed that there is a close relationship between green tea consumption and the prevention of both cancer development and

cardiovascular disease (Yang et al. 1993). These effects have been largely attributed to the most prevalent polyphenol contained in green tea, (-)-epigallocatechin gallate (EGCG). Recently, EGCG has been shown to inhibit the activity (Makimura et al. 1993; Garbisa et al. 1999; Demeule et al. 2000) and expression (Isemura et al. 1999; Maeda-Yamamoto et al. 2003) of collagenase or gelatinase (MMP-2 and MMP-9). Furthermore, it has been reported that EGCG could induce the apoptotic cell death of osteoclasts (Nakagawa et al. 2002).

However, the biological effect of EGCG on alveolar bone destruction has not been documented. Therefore, in order to examine whether EGCG has an the inhibitory effect on alveolar bone resorption, we evaluated the effect of EGCG on the gene expression of MMP-2, -9, and -13 in mouse calvarial primary osteoblastic (POB) cells, which were stimulated by sonicated *P.gingivalis* extracts (SPEs). We also investigated the inhibitory effect of EGCG on osteoclast formation in a co-culture system of mouse bone marrow cells and calvarial POB cells.

II. Materials and Methods

A. *P.gingivalis* culture and preparation of sonicated *P.gingivalis* extracts (SPEs)

P.gingivalis strain ATCC 33277 was cultured in a brain heart infusion (BHI) broth, which contained 5 ㎎/㎖ of hemin and 0.5 ㎎/㎖ of Vitamin K at 37℃ in an anaerobic chamber in an atmosphere containing 80% N_2, 10% H_2, and 10% CO_2. After 2 days of culture, the bacteria were harvested by centrifugation at 3,200 × g for 20 minutes at 4℃ and washed 3 times with phosphate buffered saline (PBS). The purity of the cultures was confirmed by phase-contrast microscopy and Gram staining. The bacterial culture was subjected to sonication*. The insoluble debris was removed by centrifugation at 12,000 × g for 5 minutes at 4℃. The supernatant was sterilized by filtering through a membrane filter with a pore size of 0.22 ㎛. The protein content of the SPEs was determined using a protein assay reagent kit† according to the manufacturer's instructions. The SPEs were stored at -70℃ until used. The concentration of SPEs (1 ㎍/㎖) used in the present study was based on our pilot cytotoxicity experiment.

---

* Misonix Inc., Farmingdale, NY
† Pierce, Rockford, IL

B. Preparation of primary osteoblastic (POB) cells and bone marrow cells

POB cells were prepared from the calvariae of 1 or 2-day-old newborn ICR mice‡ by a previously reported method (Suda et al. 1997) with a slight modification. The calvariae removed from 10 mice were subjected to digestion four times 20-minute intervals using 0.2% collagenase§ and 0.1% dispase** in a shaking water bath at 37℃. The POB cells isolated in the first digestion were discarded, and those in the second to fourth digestions were collected and cultured to confluence in an α-minimum essential medium (α-MEM)** containing 10% fetal bovine serum (FBS)** and antibiotic-antimycotic (100 units/㎖ penicillin G sodium, 100 ㎍/㎖ streptomycin sulfate and 0.25 ㎍/㎖ amphotericin B)** in a 10 cm culture dish at 37℃ with 5% CO_2. The cells were then detached from the culture dish by treating them with trypsin-EDTA ** and collected by centrifugation. The bone marrow cells were collected from the femurs and tibiae of 4 to 8-week-old ICR mice. The ends of femurs and tibiae were removed and the marrow cavity was flushed by slowly injecting media in at one end using a 25 gauge needle. The collected bone marrow cells were washed, and treated with 10 mM Tris-HCl, 0.83% ammonium chloride to remove the red blood cells.

---

‡ Samtako Inc., O-San, Kyung-gi-Do, Korea
§ Wako Pure Chemical Industries, Ltd., Osaka, Japan
** GibcoBRL, Life Technologies, Grand Island, NY, USA

C. Cytotoxicity assay

The MTT colorimetric assay was used to measure the viability of the cells after treatment with EGCG. The number of viable cells was determined based on the reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dipheyltetrazolium bromide) dye†† by mitochondria dehydrogenase in live cells to form blue formazan crystals (Mosmann 1983). POB cells (10^4 cells/well) were seeded in 96-well plates and grown in α-MEM containing 10% FBS to sub-confluence. The cells were then treated with various concentrations of EGCG‡‡ for an additional 3 days. In addition, after POB cells (5×10^3 cells/well) had been co-cultured with the bone marrow cells (5×10^4 cells/well) in α-MEM containing 10% FBS in 96-well plates for 3 days, various concentrations of EGCG were added to each well and the cells cultured for an additional 3 days. After 3 days of culture, 50 ㎕ of MTT solution (5 ㎎/㎖) was added to each well and the cells incubated for 4 hours at 37℃. The supernatant was discarded and 200 ㎕ of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. The optical density of the formazan solution was measured at 570 ㎚.

---

†† Sigma Chemical Co., St. Louis, MO
‡‡ Calbiochem, La Jolla, CA

D. Reverse transcription-polymerase chain reaction (RT-PCR)

POB cells were seeded in 35 ㎜ culture dishes at a density of 2 × 10^5 cells/dish and grown to confluence in α-MEM containing 10% FBS. The cells were precultured for 24 h in serum-free α-MEM containing 1 ㎎/㎖ BSA** and subsequently treated at the indicated concentrations for 24 h as follows; 1) no treatment, 2) EGCG alone, 3) SPEs alone, and 4) EGCG + SPEs.

The cells were collected and total RNA was extracted from the POB cells using a reagent§§ according to the manufacturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 ㎚. RT-PCR for MMP-2, -9, -13 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was carried out with a commercial one step RNA-PCR kit***. Total RNA (1 ㎍) isolated from each sample was used as a template for the cDNA synthesis. The reverse transcription of total RNA to cDNA and subsequent amplification were performed in a single tube containing 50 ㎕ of reaction mixture (Mallet et al. 1995). Each reaction tube contained 1㎍ of total RNA, 0.1 unit/㎕ of Avian Myeloblastosis Virus (AMV) RTase, 0.1 unit/㎕ of *Taq* DNA polymerase, 0.8 unit/㎕ RNase inhibitor, 5 mM $MgCl_2$, 1mM dNTPs, and 0.4 μM of each forward and reverse oligonucleotide primer (Table 1) in a one step RNA PCR buffer. RT-PCR was performed in a thermocycler†††. The cycling conditions were 30 min at 50℃ (reverse transcription) and 2 min at 94℃ (reverse transcriptase inactivation), and then 35 cycles of standard PCR were performed with each cycle consisting of 30 sec at 95℃, 45 sec at 60℃ and 1 min at 7 2℃. After amplification, 10 ㎕ of each PCR product was analyzed by 1.5%

§§ TRIzol; Gibco BRL, Life Technologies
*** Takara Shuzo co., Ltd.
††† Tgrdient, Biometra, Germany

agarose gel electrophoresis. The density of the bands was computer-analyzed by Tina Image software‡‡‡. All the data were normalized according to the GAPDH mRNA level. The density ratios of MMP to GAPDH were calculated and then represented in the form of graphs.

Table 1. Synthetic oligonucleotide primers used for RT-PCR

Target gene	Primer sequence	Length of PCR product (base pairs)
MMP-2*	Forward: 5'-CTGTCCTGACCAAGGATATAGCCT-3' Reverse: 5'-ACCTGTGGGCTTGTCACGTGGTGT-3'	355
MMP-9*	Forward: 5'-CTGTCCAGACCAAGGGTACAGCCT-3' Reverse: 5'-GTGGTATAGTGGGACACATAGTGG-3'	263
MMP-13*	Forward: 5'-CATTCAGCTATCCTGGCCACCTTC-3' Reverse: 5'-CAAGTTTGCCAGTCACCTCTAAGC-3'	250
GAPDH*	Forward: 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' Reverse: 5'-CATGTAGGCCATGAGGTCCACCAC-3'	983

* Uchida et al. 2000

‡‡‡ Raytest, Wilmington, NC, USA

E. *In vitro* formation of osteoclasts

POB cells (10^4 cells/well) were co-cultured with bone marrow cells (10^5 cells/well), using a previously described procedure (Choi et al. 2001), in 48-well plates (200 ㎕/well) in α-MEM containing 10% FBS. All cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air. EGCG or $1,25(OH)_2D_3$ (10^{-8} M)†† were added to the co-culture immediately after exchanging the medium on day 3. After an additional 4 days, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), an enzyme generally accepted as a marker for osteoclasts (Minkin 1982), using an acid phosphatase kit†† . TRAP-positive multinucleated cells showing more than 3 nuclei were considered to be osteoclasts, and were counted as such.

F. Statistical analysis

Data were expressed as means ± standard deviation (S.D.). Statistical differences were determined by analysis of variance (ANOVA) using the SAS 8.02 program. Tukey's test was used for the *post hoc* comparison of specific groups. Statistical significance was determined at the $P<0.05$ level.

III. Results

A. Effect of EGCG on cell viability

The MTT assay was performed to assess the effect of EGCG on cell viability of EGCG and to determine the appropriate concentration to be used for the treatment of the cells. EGCG showed no effect on the viability of POB cells or co-culture cells up to a concentration of 20 μM after 3 days of treatment, as compared with the non-treated cells (Fig. 1). However, inhibition of cell growth was observed at the higher concentrations of EGCG (50 μM and 100 μM for the POB cells and co-culture cells, respectively). Therefore, EGCG was used at a concentration of less than 20 μM in the subsequent studies.

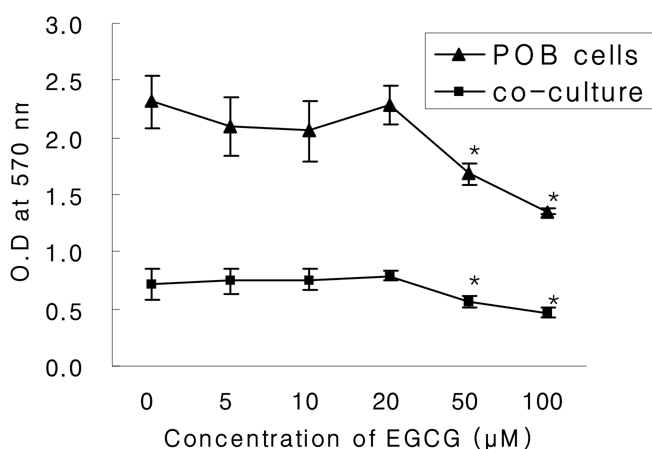
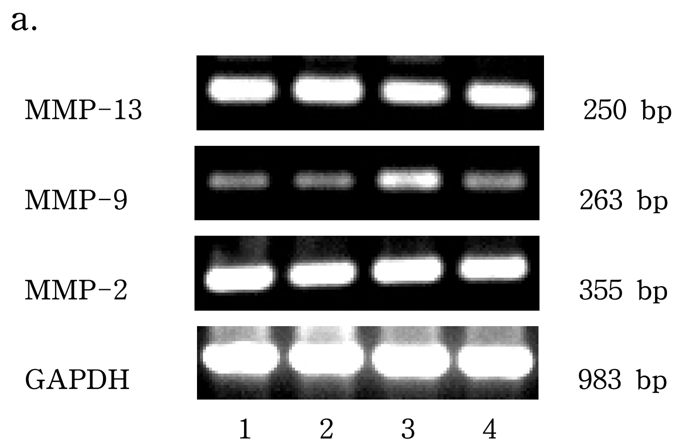


Figure1. The effect of EGCG on the viability of POB cells and the co-culture. POB cells and a co-culture of POB cells and bone marrow cells were treated with various concentrations of EGCG for 3 days. The cellular activity was then estimated by MTT assay, and the results are expressed as the mean ± SD of six cultures. The data are representative of three separate experiments. *$P<0.05$; significantly different from the non-treated group

B. Effect of SPEs and EGCG on the expression of MMP-2, -9, and -13 mRNA in POB cells

Mouse calvarial primary osteoblastic (POB) cells were cultured to confluence. At confluence, the cells were treated for 24 h as follows; 1) no treatment, 2) 20 μM of EGCG alone, 3) 1 ㎍/㎖ of SPEs alone, and 4) 20 μM of EGCG + 1 ㎍/㎖ of SPEs. As shown in Fig. 2a, the expression of MMP-2, -9, and -13 was detected in all treatment groups by RT-PCR analysis. The treatment with SPEs (1 ㎍/㎖) stimulated the expression of MMP-9 mRNA, showing a 215% increase in the density ratio analysis when compared to the untreated cells. In addition, this effect was significantly inhibited by EGCG (20 μM), reaching the same level of the expression of MMP-9 mRNA as that observed in the untreated cells. However, EGCG alone had no effect on the expression of MMP-9 mRNA, and the expression of MMP-2 and MMP-13 mRNA was almost never affected by SPEs and EGCG (Fig. 2b).



b.

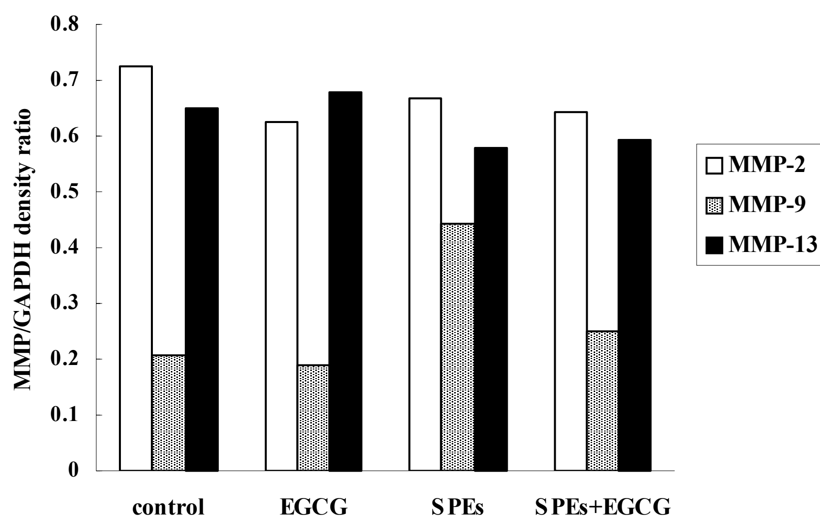
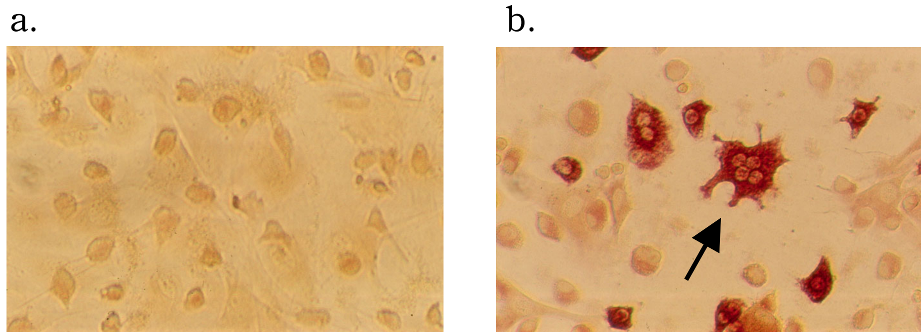


Figure 2. The effect of SPEs and EGCG on the expression of MMP-2, -9, and -13 mRNA. POB cells were treated for 24 h with; 1) no treatment, 2) 20 µM of EGCG alone, 3) 1 ㎍/㎖ of SPEs alone, and 4) 20 µM of EGCG + 1 ㎍/㎖ of SPEs. The expression of MMP-2, -9, and -13 mRNA was analyzed by RT-PCR (a). The density ratios of MMPs to GAPDH are represented in graph (b). The data are representative of experiments.

C. Inhibitory effect of EGCG on osteoclast formation

To examine the effect of EGCG on osteoclast formation, the co-culture of POB cells and bone marrow cells was treated with various concentrations of EGCG in the presence of $1,25(OH)_2D_3$ (10^{-8} M). $1,25(OH)_2D_3$ was used as a positive control to induce the formation of osteoclasts. The largest number of TRAP-positive osteoclasts was detected following the treatment with EGCG in the presence of $1,25(OH)_2D_3$ (10^{-8} M) after 4 days of culture (Fig. 3a). α-MEM containing 10 % FBS was used as a negative control. No TRAP-positive osteoclasts were formed in the negative control (Fig. 3b). The addition of 20 μM EGCG to the co-culture resulted in a significant decrease in the number of osteoclasts to approximately 32% of the level of the positive control. In addition, the effect of EGCG on the viability of the co-culture was re-evaluated by MTT assay on the duplicate 48-well culture plate. The MTT assay confirmed that EGCG had no cytotoxic effect on the co-culture at a lower concentrations lower than 20 μM (Fig. 3c).



c.

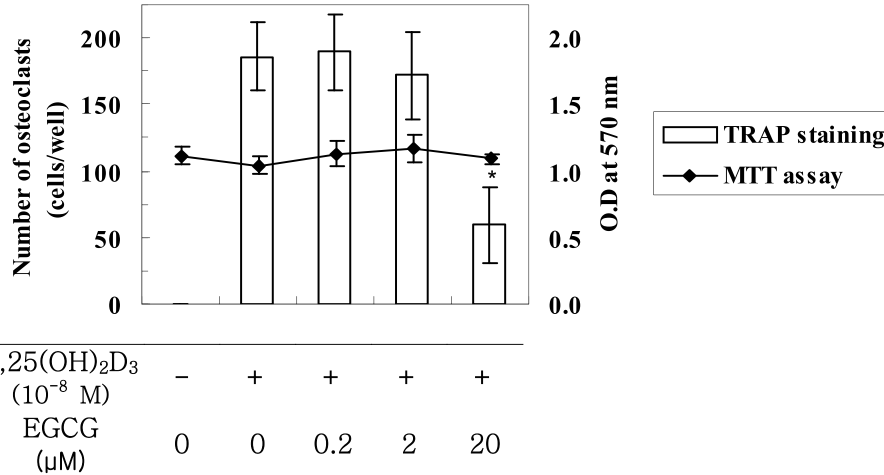


Figure 3. The effect of EGCG on osteoclast formation. Mouse bone marrow and POB cells were co-cultured for 3 days. After changing the medium, the cells were treated with various concentrations of EGCG for an additional 4 days in the presence of $1,25(OH)_2D_3$ (10^{-8} M). Non-treated cells (a) or $1,25(OH)_2D_3$ treated cells (b) without EGCG were used as the negative and positive control, respectively (original magnification × 200). After co-culture, cells were fixed and stained for TRAP. Arrow indicates TRAP-positive multinucleated cell. (c) The TRAP-positive multinucleated cells with more than 3 nuclei were counted as osteoclasts. At the same time, the effect of EGCG on the viability of the co-culture was re-evaluated by MTT assay on the duplicate 48-well culture plate. The results are expressed as the mean ± SD of four cultures. The data are representative of three separate experiments. $*P<0.05$; significantly different from the positive control.

IV. Discussion

Green tea consists mainly of polyphenols (catechins) which constitute up to 30% of the dry weight. The major catechins in green tea are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), and (+)-catechin (C). Among these polyphenols, EGCG is the most abundant catechin and the one that has been the most extensively studied (Graham 1992). Numerous biological effects of green tea and its constituents have been reported. These include an anticarcinogenic activity, anti-inflammatory effect, the alleviation of cardiovascular disease, etc. (Yang et al. 1993; Krahwinkel et al. 2000). Recent studies demonstrated that EGCG inhibited the activity (Makimura et al. 1993; Garbisa et al. 1999; Demeule et al. 2000) and expression (Isemura et al. 1999; Maeda-Yamamoto et al. 2003) of collagenase or gelatinase (MMP-2 and MMP-9). Furthermore, it has been reported that EGCG could induce the apoptotic cell death of osteoclasts (Nakagawa et al. 2002). In periodontitis, periodontal tissues are continuously challenged by periodontopathogens, such as *P.gingivalis*. It was shown that *P.gingivalis* could mediate the destruction of periodontal tissue and could be involved in alveolar bone resorption in various ways (Millar et al. 1986; Bom-van Noorloos et al. 1990; Loomer et al. 1994; Kadono et al. 1999; Jiang et al. 2002). In addition, the products of *P.gingivalis* were reported to have the ability to induce and activate host MMPs (Uitto et al. 1989; Sorsa et al. 1992; Fravalo et al. 1996; DeCarlo et al. 1997; Chang et al. 2002; Grayson et al. 2003; Pattamapun et al. 2003). However, the effects of EGCG and *P.gingivalis* on MMPs listed above have not been examined in osteoblastic cells. Moreover, no findings have been reported for the effect of EGCG on the gene expression of MMPs in osteoblasts.

These findings led us to investigate the effect of EGCG and *P.gingivalis* on the expression of MMPs in osteoblasts. We also examined the effect of EGCG on the formation of osteoclasts using a co-culture of POB cells and bone marrow cells.

In the present study, we showed by RT-PCR analysis that SPEs stimulated the expression of MMP-9 mRNA in mouse calvarial POB cells and that EGCG (20 μM) significantly inhibited this stimulatory effect (Fig. 2). Since EGCG alone did not affect the transcription level of MMP-9, we concluded that the effect of EGCG was limited to preventing the increase of MMP expression induced by SPEs. These findings suggest that *P.gingivalis* may contribute to the alveolar bone loss observed in periodontal disease, by stimulating host osteoblastic cells to produce MMP. In addition, this inhibitory effect of EGCG indicates the potential of EGCG to prevent the bone resorption caused by periodontal pathogens, such as *P.gingivalis*. On the other hand, although the mRNA of MMP-2 and MMP-13 appeared to be expressed in POB cells, as shown in previous studies (Uchida et al. 2000), the transcription levels of these MMPs were not affected by either SPEs or EGCG. In a recent study, the molecular basis for the EGCG-mediated regulation of MMP-2 and MMP-9 was investigated in cancer cells (Maeda-Yamamoto et al. 2003). This study suggested that EGCG caused the reduction in MMP activities by inhibiting the gene expression of MMP-2 and MMP-9 by suppressing the phosphorylation of extracellular signal-regulated kinase (ERK) in cancer cells. This protein kinase belongs to the mitogen-activated protein kinase (MAPK) family of enzymes, which is known to regulate MMP expression (Gum et al. 1997; Lim et al. 1998; McCawley et al. 1999). In a previous study, it was reported that the inhibition of MAPK suppressed MMP expression (Reddy et al. 1999). Furthermore, several studies have provided evidence suggesting that EGCG inhibits MAPK, resulting in the down-regulation of MMPs (Chung et al. 2001;

Katiyar et al. 2001). It was also reported that EGCG inhibits MAPK signal transduction to the nucleus, by preventing the association of Raf-1 with MEK1, as well as the phosphorylation of Elk-1 by Erk1/2 (Chung et al. 2001). In addition, it was found that [^{3}H]EGCG was incorporated into the cytosol as well as the nuclei (Okabe S et al. 1997). Our finding is basically consistent with these results. Therefore, it may be inferred that EGCG inhibits the stimulatory effect of SPEs on MMP expression by intracellulary blocking the MAPK signaling pathway. However, because of the large gaps in our knowledge of the mechanisms underlying the inhibition of MMP by EGCG, more extensive studies are needed to obtain conclusive evidence. Therefore, it remains to be determined exactly how EGCG influences the MAPK signal transduction pathway

We also found that EGCG inhibited the osteoclast formation induced by $1,25(OH)_2D_3$ (10^{-8} M), based on TRAP staining in the co-culture system. In the present study, EGCG remarkably reduced the number of TRAP-positive multinucleated cells at a concentration of 20 µM (Fig. 3). Although we have no ready explanation for the mechanism underlying the inhibitory effect of EGCG on osteoclast formation at present, in a previous study it was reported that EGCG inhibited bone resorption by inducing apoptotic cell death of osteoclast-like multinucleated cells in a dose-dependent manner (25-100 µM) without any significant effect on osteoblastic cells (Nakagawa et al. 2002). In addition, various studies have reported that EGCG induces apoptosis in tumor cells (Ahmad et al. 1997; Yang et al. 1998; Paschka et al. 1998). However, in the present study, we found that EGCG had no inhibitory effect on the cell viability of either the co-culture system or POB cells, at concentrations of up to 20 µM. Therefore, the observed reduction in the number of osteoclasts is thought to be mediated via a mechanism other than apoptosis. Therefore, the precise mechanism of action of EGCG on osteoclasts remains to be determined.

In addition, since the bone resorbing activity of osteoclasts plays a crucial role in bone resorption, EGCG, with its ability to inhibit the formation of osteoclasts, might have the potential to be used in the treatment of bone diseases such as periodontitis. In addition, the low toxicity of EGCG and its distribution in bone tissue, as shown previously (Suganuma et al. 1998), supports its effectiveness *in vivo*. However, because we examined only the *in vitro* effects of EGCG in the present study, it remains to be determined whether EGCG exerts these effects *in vivo*.

In conclusion, in this study, we have shown that EGCG has an inhibitory effect on the gene expression of MMP-9 in osteoblasts and on the formation of osteoclasts. These findings suggest that EGCG may inhibit bone resorption by preventing the action of osteoblasts and osteoclasts. Therefore, our data indicate that the inhibitory activity of EGCG could be usefully applied to the development of a therapeutic agent for the treatment of bone diseases such as periodontitis.

V. Conclusion

The purpose of the present study was to examine whether EGCG could have the inhibitory effect on alveolar bone resorption. For this purpose, we evaluated the effects of green tea polyphenol EGCG on the gene expression of osteoblast-derived MMP-2, -9, and -13, stimulated by *P.gingivalis*, and on the formation of osteoclasts.

The effect of EGCG on the gene expression of MMPs was examined by treating mouse calvarial POB cells with EGCG (20 μM) in the presence of SPEs. The transcription levels of MMP-2, -9, and -13 were assessed by RT-PCR. The effect of EGCG on osteoclast formation was confirmed by TRAP staining in a co-culture system of mouse bone marrow cells and calvarial POB cells. The results were as follows.

1. Treatment with SPEs stimulated the expression of MMP-9 mRNA and this effect was significantly reduced by EGCG (20 μM). However, EGCG alone had no effect on the expression of MMP-9 mRNA, and the expression of MMP-2 and MMP-13 mRNA was almost never affected by SPEs and EGCG

2. EGCG significantly inhibited the osteoclast formation in the co-culture system at a concentration of 20 μM.

The present study shows that EGCG has an inhibitory effect on the gene expression of MMP-9 in osteoblasts and on the formation of osteoclasts. These findings suggest that EGCG may inhibit bone resorption by preventing the action of osteoblasts and osteoclasts. Therefore, our data indicate that the

inhibitory activity of EGCG could be usefully applied to the development of a therapeutic agent for the treatment of bone diseases such as periodontitis.

VI. Reference

Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3 gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *JNCI* 89:1881-1886, 1997.

Baron R. Molecular mechanisms of bone resorption by the osteoclast. *Anat Rec* 224:317-324, 1989.

Birkedal-Hansen H. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 64:474-484, 1993.

Birkedal-Hansen H. Role of cytokines and inflammatory mediators in tissue destruction. *J Periodont Res* 28:500-510, 1993.

Blair HC, Teitelbaum SL, Ghiselli R, Gluck S. Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* 245:855-857, 1989.

Bom-van Noorloos AA, van der Meer JW, van de Gevel JS, Schepens E, van Steenbergen TJ, Burger EH. *Bacteroides gingivalis* stimulates bone resorption via interleukin-1 production by mononuclear cells. *J Clin Periodontol* 17:409-413, 1990.

Chambers TJ, Darby JA, Fuller K. Mammalian collagenase predisposes bone surfaces to osteoclastic resorption. *Cell Tiss Res* 241:671-675, 1985.

Chambers TJ, Fuller K. Bone cells predispose bone surfaces to resorption by exposure of mineral to osteoclastic contact. *J Cell Sci* 76:155-165, 1985.

Chang YC, Yang SF, Lai CC, Liu JY, Hsieh YS. Regulation of matrix metalloproteinase production by cytokines, pharmacological agents and periodontal pathogens in human periodontal ligament fibroblast cultures. *J Periodont Res* 37:196-203, 2002.

Choi BK, Ohk SH, Lee HJ, Kang JH, Jeong GJ, Yoo YJ. Effects of whole cell sonicates of *Treponema lecithinolyticum* on osteoclast differentiation. *J Periodontol* 72:1172-1177, 2001.

Chung JY, Park JO, Phyu H, Dong Z, Yang CS. Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (−)-epigallocatechin-3-gallate and theaflavin-3,3′-digallate. *FASEB J* 15:2022-2024, 2001.

DeCarlo AA, Windsor LJ, Bodden MK, Birkedal-Hansen B, Birekdal-Hansen H. Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe *Porphyromonas gingivalis*. *J Dent Res* 76:1260-1270, 1997.

Delaisse JM, Engsig MT, Everts V, del Carmen Ovejero M, Ferreras M, Lund L, Vu TH, Werb Z, Winding B, Lochter A, Karsdal MA, Troen T, Kirkegaard T, Lenhard T, Heegaard AM, Neff L, Baron R, Foged NT. Proteinases in bone resorption: obvious and less obvious roles. *Clin Chim Acta* 291:223-234, 2000.

Demeule M, Brossard M, Page M, Gingras D, Beliveau R. Matrix metalloproteinase inhibition by green tea cathechins. *Biochim Biophys Acta* 1478:51-60, 2000.

Dzink JL, Socransky SS, Haffajee AD. The predominant cultivale microbiota of active and inactive lesions of destructive periodontal diseases. *J Clin Periodontol* 15:316-323, 1988.

Fravalo P, Mnard C, Bonnaure-Mallet M. Effect of *Porphyromonas gingivalis* on epithelial cells MMP-9 typeⅣ collagenase production. *Infect Immun* 64:4940-4945, 1996.

Garbisa S, Biggin S, Cavallarin N, Sartor L, Benelli R, Albini A. Tumor invasion: molecular shears blunted by green tea. *Nature Med* 4:12-16, 1999.

Graham HN. Green tea composition, consumption, and polyphenol chemistry. *Prev Med* 21:334-350, 1992.

Grayson R, Douglas CW, Heath J, Rawlinson A, Evans GS. Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and

Porphyromonas gingivalis. *J Clin Periodontol* 30:542-550, 2003.

Gum R, Wang H, Lengyel E, Juarez J, Boyd D. Regulation of 92kDa type Ⅳ collagenase expression by the *jun* aminoterminal kinase- and the extracellular signal-regulated kinase-dependent signaling cascades. *Oncogene* 14:1481-1493, 1997.

Heath JK, Atkinson SJ, Meikle MC, Reynolds JJ. Mouse osteoblasts synthesize collagenase in response to bone resorbing agents. *Biochim Biophys Acta* 802:151-154, 1984.

Hill PA. Docherty AJ, Bottomley KM, O'Connell JP, Morphy JR, Reynolds JJ, Meikle MC. Inhibition of bone resorption *in vitro* by selective inhibitors of gelatinase and collagenase. *Biochem J* 308:167-175, 1995.

Isemura M, Saeki K, Minami T, Hayakawa S, Kimura T, Shoji T, Sazuka M. Inhibition of matrix metalloproteinases by tea catechins and related polyphenols. *Ann N Y Acad Sci* 878:629-631, 1999.

Jiang Y, Mehta CK, Hsu TY, Alsulaimani FF. Bacteria induce osteoclastogenesis via an osteoblast-independent pathway. *Infect Immun* 70:3143-3148, 2002.

Kadono H, Kido J, Kataoka M, Yamauchi N, Nagata T. Inhibition of osteoblastic cell differentiation by lipopolysaccharide extract from *Porphyromonas gingivalis*. *Infect Immun* 67:2841-2846, 1999.

Katiyar SK, Afaq F, Azizuddin K, Mukhtar H. Inhibition of UVB-Induced oxidative stress-mediated phophorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (-)-epigallocatechin-3-gallate. *Toxicol Appl Phamacol* 176:110-117, 2001.

Krahwinkel T, Willershausen B. The effect of sugar-free green tea chew candies on the degree of inflammation of the gingiva. *Eur J Med Res* 30:463-467, 2000.

Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, Kamoi K, Suda T. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. *Endocrinology* 139:1338-1345, 1998.

Lim M, Martinez T, Jablons D, Cameron R, Guo H, Toole B, Li JD, Basbaum C. Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38. *FEBS Lett* 441:88-92, 1998.

Loomer PM, Sigusch B, Sukhu B, Ellen RP, Tenenbaum HC. Direct effects of metabolic products and sonicated extracts of *Porphyromonas gingivalis* 2561 on osteogenesis *in vitro*. *Infect Immun* 62:1289-1297, 1994.

Maeda-Yamamoto M, Suzuki N, Sawai Y, Miyase T, Sano M, Hashimoto-Ohta A, Isemura M. Association of suppression of extracellular signal-regulated kinase phosphorylation by epigallocatechin gallate with the reduction of matrix metalloproteinase activities in human fibrosarcoma HT1080 cells. *J Agric Food Chem* 51:1858-1863, 2003.

Makimura M, Hirasawa M, Kobayashi K, Indo J, Sakanaka S, Taguchi T, Otake S. Inhibitory effect of tea catechins on collagenase activity. *J Periodontol* 64:630-636, 1993.

Mallet F, Oriol G, Mary C, Verrier B, Mandrand B. Continuous RT-PCR using AMV-RT and *Taq* DNA polymerase: characterization and comparison to uncoupled procedures. *BioTechniques* 18:678-687, 1995.

McCawley LJ, Li S, Wattenberg EV, Hudson LG. Sustained activation of the mitogen-activated protein kinase pathway. *J Biol Chem* 274:4347-4353, 1999.

Meikle MC, Bord S, Hembry RM, Compston J, Croucher PI, Reynolds JJ. Human osteoblasts in culture synthesize collagenase and other matrix metalloproteinases in response to osteotropic hormones and cytokines. *J Cell Sci* 103:1093-1099, 1992.

Millar SJ, Goldstein EG, Levine MJ, Hausmann E. Modulation of bone metabolism by two chemically distict lipopolysaccharide fractions from *Bacteroides gingivalis*. *Infect Immun* 51:302-306, 1986.

Minkin C. Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif Tissue Int* 34:285-290, 1982.

Mizutani A, Sugiyama I, Kuno E, Matsunaga S, Tsukagoshi N. Expression of matrix metalloproteinases during ascorbate-induced differentiation of osteoblastic MC3T3-E1 cells. *J Bone Miner Res* 16:2043-2049, 2001.

Moore WE. Microbiology of periodontal disease. *J Periodont Res* 22:335-341, 1987.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63, 1983.

Nakagawa H, Wachi M, Woo JT, Kato M, Kasai S, Takahashi F, Lee IS, Nagai K. Fenton reaction is primarily involved in a mechanism of (-)-epigallocatechin-3-gallate to induce osteoclastic cell death. *Biochem Biophys Res Commun* 288:142-150, 2002.

Okabe S, Suganuma M, Hayashi M, Sueoka E, Komori A, Fujiki H. Mechanism of growth inhibition of human lung cancer cell line, PC-9, by tea polyphenols. *Jpn J Cancer Res* 88:639-643, 1997.

Paschka AG, Butler R, Young CY. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3 gallate. *Cancer Lett* 130:17, 1998.

Pattamapun K, Tiranathanagul S, Yongchaitrakul T, Kuwatanasuchat J, Pavasant P. Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells. *J Periodont Res* 38:115-121, 2003.

Reddy KB, Krueger JS, Kondapaka SB, Diglio CA. Mitogen-activated protein kinase (MAPK) regulates the expression of progelatinase B (MMP-9) in

breast epithelical cells. *Int J Cancer* 82:268-273, 1999.

Reynolds JJ, Hembry RM, Meikle MC. Connective tissue degradation in health and periodontal disease and the roles of matrix metalloproteinases and their natural inhibitors. *Adv Dent Res* 8:312-319, 1994.

Reynolds JJ, Meikle MC. Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontol* 2000 144-157, 1997.

Schwartz Z, Goultschin J, Dean DD, Boyan BD. Mechanisms of alveolar bone destruction in periodontitis. *Periodontol* 2000 141:158-172, 1997.

Slots J, Listgarten MA. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans*in human periodontal diseases . *J Clin Periodontol* 15:85-93, 1988.

Sorsa T, Ingman T, Suomalainen K, Haapasalo M, Konttinen YT, Lindy O, Saari H, Uitto VJ. Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. *Infect Immun* 60:4491-4495, 1992.

Suda T, Jimi E, Nakamura I, Takahashi N. Role of 1,25-dihydoxyvitamin D_3 in osteoclast differentiation and function. *Methods Enzymol* 282:223-235, 1997.

Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H, Fujiki H. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* 19:1771-1776, 1998.

Uchida M, Shima M, Shimoaka T, Fujieda A, Obara K, Suzuki H, Nagai Y, Ikeda T, Yamato H, Kawaguchi H. Regulation of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) by bone resorptive factors in osteoblastic cells. *J Cell Physiol* 185:207-214, 2000.

Uitto VJ, Larjava H, Heino J, Sorsa T. A protease of *Bacteroides gingivalis* degrades cell surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretion of collagenase and plasminogen activator. *Infect Immun* 57:213-218, 1989.

Yang CS, Wang ZY. Tea and cancer. *J Natl Cancer* 85:1038-1049, 1993.

Yang G, Liao J, Kim K, Yurkow EJ, Yang CS. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* 19:611-616, 1998.

국문 요약

녹차 polyphenol (-)-epigallocatechin gallate (EGCG)의 matrix metalloproteinase-9 발현과 파골세포 분화 억제효과

연세대학교 대학원 치의학과

(지도 최성호 교수)

윤 정 호

치조골 흡수는 치주질환의 특징적인 양상이며, 골의 무기질과 유기질의 제거가 일어난다. 이러한 과정에는 파골세포와 matrix metalloproteinase (MMP)가 관여한다. 그람 음성 세균인 *Porphyromonas gingivalis*는 여러 MMP의 활성과 발현을 자극하는 것으로 보고 되었다. 한편, (-)-epigallocatechin gallate (EGCG)는 녹차의 주된 성분으로서 여러 MMP의 활성과 발현에 억제 효과를 지니고 있는 것으로 보고 되었다.

본 연구에서는 *P.gingivalis*에 의해 자극된 조골세포의 MMP-2, -9, -13 유전자 발현에 미치는 EGCG의 영향이 조사되었다. 또한, EGCG가 파골세포 분화에 미치는 영향도 조사되었다. EGCG가 MMP 유전자 발현에 미치는 영향은 마우스 두개골 일차 조골세포를 *P.gingivalis* 세균 분쇄액 (SPEs)과 EGCG (20 μM)로 처리함으로써 조사되었다. MMP-2, -9, -13의 mRNA 양은 reverse transcription-polymerase chain reaction (RT-PCR)에 의해서 규명되었으며, 파골세포 형성에 미치는 EGCG의 효과는 마우스 골수 세포와 두개골 일차 조골세포를 이용한 co-culture system에서 tartrate-resistant acid phosphatase

(TRAP) 염색에 의해 확인되었다.

SPEs의 처리는 MMP-9 mRNA의 발현을 상당히 증가시켰으며 ($P<0.05$), 이러한 효과는 EGCG에 의해서 유의성 있게 억제되었다 ($P<0.05$). 반면에 MMP-2와 MMP-9 mRNA의 발현은 SPEs와 EGCG에 의해서 영향받지 않았다. 또한, EGCG는 co-culture system에서의 파골세포 형성을 20 μM의 농도에서 대조군과 비교하여 크게 억제 하였다 ($P<0.05$).

이상의 결과에서 볼 때, EGCG가 조골세포에서의 MMP-9 유전자 발현과 파골세포 형성을 억제하므로 치주질환에서 치조골 흡수를 억제할 수 있을 것으로 사료되며, 이러한 EGCG의 억제 작용은 치주염 같은 골 질환의 치료를 위한 치료제 개발에 유용하게 적용될 수 있을 것이다.

핵심되는말 : matrix metalloproteinase, (-)-epigallocatechin gallate (EGCG), 조골세포, 파골세포