The effect of epigallocatechin-3-gallate on the osteogenic differentiation of human alveolar bone cells both *in vitro* and *in vivo*

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Directed by Professor Hyung-Jun Choi

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Yon Joo Mah

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This certifies that the dissertation of Yon Joo Mah is approved.

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Yonsei University

August 2013

감사의 글

오늘 박사 논문을 마무리하며 이 논문이 끝날 수 있도록 물심 양면으로 도와주시고 이끌어주신 분들께 깊은 감사의 마음을 전하고 싶습니다.

수련 과정 수료 후 이렇게 박사 과정의 마지막까지 하는 동안 많은 격려를 해주신 손흥규 교수님, 소아치과 의사로서의 기본을 가르쳐주신 최병재 교수님, 앞으로 나아가야 할 방향을 같이 고민해주신 이제호 교수님, 지도 교수가 되어 주시고 부족한 저를 인자함으로 이끌어주신 최형준 교수님, 항상 반가운 얼굴로 맞아주시고 끝없는 격려를 해주신 김성오 교수님, 그리고 연구에 눈을 뜰 수 있도록 이끌어 주시고 같이 고민하고 공부하고 마지막까지 잘 마무리할 수 있도록 여러 방면으로 도와주신 송제선 교수님께 깊은 감사를 전합니다.

연구를 진행함에 있어 많은 자료들로 도움을 주신 문석준 교수님과 실험 과정의 진행과 좀 더 좋은 논문이 나올 수 있도록 도움이 되는 많은 말씀을 해주신 정의원 교수님께 감사를 드립니다.

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그리고 지금까지 제가 공부를 지속할 수 있도록 깊은 배려와 사랑으로 응원해 주신 부모님과 가족들과 지치고 힘들어할 때 항상 곁에서 응원해준 동기인 정은언니와 승혜언니에게도 감사를 드립니다. 부족한 제가 이 논문을 쓸 수 있었던 것은 많은 분들의 도움과 헌신이 있었기에 가능한 일이었습니다. 감사합니다.

2013년 6월

마연주 드림

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The effect of epigallocatechin-3-gallate on the osteogenic differentiation of human alveolar bone cells both *in vitro* and *in vivo*

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(Directed by Professor Hyung-Jun Choi)

The effects of epigallocatechin-3-gallate (EGCG), a major catechin in green tea, on human and mouse osteoblasts remain controversial. This study investigated the direct effects of EGCG on human alveolar bone-derived cells (hABCs) both *in vitro* and *in vivo*.

hABCs were treated with EGCG at various concentrations, and a proliferation assay, flow cytometric analysis for apoptosis evaluation, migration assay, and *in vitro*

osteogenic differentiation were performed. hABCs that were pretreated with 10 μM EGCG and mixed with calcium phosphate carrier combined with EGCG *in vivo* were transplanted into immunodeficient mouse. Histological staining, quantitative gene expressions, and alkaline phosphatase activity were evaluated in the retrieved transplants.

The proliferation and migration were decreased when EGCG was present at over 25 μM. The osteogenic differentiation increased slightly when EGCG was present at up to 10 μM, and clearly decreased for higher concentrations of EGCG. *In vivo*, the potential for hard-tissue formation was slightly higher for the group with 0.1 mg of EGCG than for the control group, and decreased sharply for higher concentrations of EGCG.

The present observations suggest that EGCG at a low concentration could not enhance the osteogenic effect, whereas at a higher concentration it could prevent the osteogenic differentiation of hABCs both *in vitro* and *in vivo*.

Keywords: alveolar bone-derived cells, epigallocatechin-3-gallate (EGCG), osteogenic differentiation, *in vivo* transplantation

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I. Introduction

Green tea is one of the most popular beverages consumed worldwide, and it contains salubrious polyphenols, in particular catechins, carotenoids, tocoperols, ascorbic acid, minerals such as chromium and manganese, and certain phytochemical compounds (Cabrera, Artacho, and Gimenez 2006, Singh, Akhtar, and Haqqi 2010). (-)-Epigallocatechin-3-gallate (EGCG) accounts for more than 50% of the total catechin

content in green tea (up to 63%) (Cabrera, Artacho, and Gimenez 2006, Ko et al. 2009, Shen et al. 2011), and green tea catechins in general exert many beneficial effects on human health, including reducing blood pressure (Yang YC 2004), decreasing serum lipids (Moon et al. 2007), and having antitumorigenic (Kim, Amin, and Shin 2010), anti-inflammatory (Singh, Akhtar, and Haqqi 2010), anticariogenic (Otake et al. 1991), antiaging (Mandel et al. 2008), and antioxidant (Na, and Surh 2008) effects.

Recent researches have suggested that bone mineral density is positively associated with green tea consumption (Hegarty, May, and Khaw 2000, Wu et al. 2002). In bone metabolism it has been shown that EGCG suppresses bone resorption (Delaisse, Eeckhout, and Vaes 1986, Lin et al. 2009, Nakagawa et al. 2002, Yun et al. 2007, Yun et al. 2004) and inhibits the formation of osteoclasts *in vitro* (Morinobu et al. 2008, Shen et al. 2008b, Yun et al. 2004). Although there is considerable evidence that EGCG suppresses osteoclast activity, few studies (Rodriguez et al. 2011, Vali, Rao, and El-Sohemy 2007) have considered the direct dose-dependent effects of EGCG on osteoblast differentiation or bone formation. And there are many researches that suggest drinking green tea of proper concentration with animal models should induce the periosteal bone formation and increase bone mineral density (Shen et al. 2008a, Shen et al. 2010, Shen et al. 2009), and that locally injection into infected gingival and contact with dentifrice directly could reduce alveolar bone resorption with decreasing osteoclastogenesis and interrupt inflammation (Maruyama et al. 2011, Nakamura et al. 2010)

There are conflicting reports on the effects of EGCG on osteoblasts *in vitro* and *in vivo*. A recent study found that EGCG increases the formation of mineralized bone

nodules and alkaline phosphatase (ALP) activity in human osteosarcoma SaOS-2 cells (Vali, Rao, and El-Sohemy 2007). In addition, an *in vivo* experiment showed that EGCG could increase the bone regenerative capacity in a rat calvarial bone defect model (Rodriguez et al. 2011). However, another study found that EGCG suppressed the differentiation of murine osteoblastic MC3T3-E1 cells and decreased ALP activity (Kamon, Zhao, and Sakamoto 2010). Similarly, EGCG decreased ALP activity and calcium content in a bone morphogenetic protein (BMP)-induced ectopic bone formation model (Takita et al. 2002). EGCG could enhance bone mineralization through the Runt-related transcription factor 2 (Runx2) - mediated mechanism and it regulated differentiation of osteoblast cells (Komori et al. 1997). The application of EGCG was reported to increase the mRNA expressions of Runx2, osterix, osteocalcin (OC), and ALP (Chen et al. 2005). According to these studies, increasing of Runx2, bone sialoprotein (BSP), osteopontin (OPN) level indicated mineralization and osteoblastic differentiation (Chen et al. 2005, Komori et al. 1997).

This controversial situation prompted the present study to investigate the direct dose-dependent effects of EGCG on the human alveolar bone-derived osteoblast cells *in vitro* and *in vivo* and to assess the possibility of its clinical use in alveolar bone regeneration..

II. Materials and Methods

1. Cell Culture

Human alveolar bone tissues were collected during surgical extraction of impacted supernumerary teeth from eight children (aged 7–9 years, seven males and one female), under guidelines approved by the Institutional Review Board of the Dental Hospital, Yonsei University. Informed consents were obtained from all of the subjects and their parents with written form (#2-2011-0046). Cells obtained from young donors–corresponding to the growth period–could have greater potential for use in the bone regeneration (Huang et al. 2005, Jiang et al. 2010, Zhou et al. 2008). Human alveolar bone-derived cells (hABCs) were obtained from the obtained tissues using an outgrowth method. Briefly, alveolar bone tissues were chopped into a size of about 1.0 mm^3 and then placed in a 60-mm culture dish (BD Falcon, Franklin Lakes, NJ, USA) until the cells grew out. The hABCs were cultured in α-minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) at 37°C in 5% CO_2. The cells from the different donors were blended at the second passage, and cultures at passages 3–5 were used for all experiments.

2. Proliferation Assay

The proliferation of the cells was measured using the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan). Briefly, the cells were plated in 24-well culture plates (BD Falcon) at a density of 500 cells/well in triplicate. The cells were treated with EGCG (#E4143, Sigma, St. Louis, MO, USA) at concentrations of 1, 5, 10, 25, and 50 μM. On every other day (i.e., days 1, 3, 5, 7, and 9), the amount of water-soluble colored formazan formed by the activity of dehydrogenases was measured using a spectrophotometer (Benchmark Plus Microplate spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

3. Flow Cytometric Analysis for Apoptosis Evaluation

Cells ($1x10^5$) were seeded onto 100-mm culture dishes in the presence of EGCG at various concentrations (0, 1, 5, 10, 25, and 50 μM). After 5 days, both floating and attached cells were harvested with trypsinization and centrifugation, cell pellets were resuspended with PBS (Invitrogen) and fixed in 70% cold ethanol (Sigma) for 1 h at 4ºC, and then they were washed with PBS (Invitrogen). After centrifugation, the cell pellets were resuspended with PBS (250 μl) containing RNase A (0.2 mg/ml, LaboPass, Sapporo, Japan) for 1h at 37ºC. The cells were stained with propidium iodide (PI; 40 μg/ml; Sigma) at 4ºC for 1 h, and the cell cycle was detected by a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). The data were analyzed with FCSExpress V4 software (De Novo Software, Los Angeles, CA, USA). Apoptotic cells were indicated as subphase G0/G1.

4. Cell Migration Assay

The migration of cells was assessed by seeding them on six-well culture plates (BD Falcon) and allowing them to grow to 90% confluency. The cells were then starved with serum-free medium for 24 h and monolayers were scratched with a 200-μl pipette tip (Axygen, Hayward, CA, USA) to create a cell-free area. Cultures were washed twice with PBS to remove cell debris and covered with α-MEM containing 1% FBS, and then EGCG was added at various concentrations (1, 5, 10, 25, and 50 μM). After 6, 30, 48, and 54 h, the percentages of recovery cell migration area were calculated with the Image J program (version 1.45, NIH, Bethesda, MD, USA).

5. *In Vitro* Osteogenic differentiation

The cells were seeded at a density of 1×10^4 cells/cm^2 in 12-well culture dishes (BDFalcon). When they had reached about 100% confluency, cultures were treated with osteogenic induction medium [growth medium as described above supplemented with 0.1 M dexamethasone (Sigma), 2 mM β-glycerolphosphate (Sigma), and 50 μM ascorbic acid 2-phosphate (Sigma)], and then EGCG was added at various concentrations EGCG (1, 5, 10, 25, and 50 μM) for 4 weeks. A control was produced by culturing cells only in osteogenic induction medium. After 4 weeks, the cells were fixed for 30 min with 10% neutral-buffered formalin (Sigma) at 4°C and stained with 2% Alizarin Red S (pH 4.2; Sigma) for 10 min at room temperature. The amounts of Alizarin Red S dye extracted by 10% cetylpyridinium chloride (Sigma) were

measured using a spectrophotometer (Benchmark Plus Microplate spectrophotometer, Bio-Rad Laboratories) at 450 nm. The changes in the gene expressions of ALP and BSP during osteogenic differentiation were evaluated with the quantitative reverse-transcription-polymerase chain reaction (RT-PCR) as described below.

6. Gene expression analysis by quantitative Reverse Transcription Polymerase Chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The integrity and concentration of extracted RNA was evaluated using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). One microgram of total RNA was reverse transcribed to synthesize cDNA with the Maxime RT premix kit (Intron Biotechnology, Seoul, Korea), which used an oligo $d(T)_{15}$ primer, according to the manufacturer's instructions. A quantitative PCR assay was performed with SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) and the ABI 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Each PCR assay was carried out in duplicate for 10 s at 95°C, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, and 72°C for 60 s. Amplification specificity was confirmed by melting-curve analysis from 60°C to 95°C. The values for each gene were normalized to the expression levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and the relative expression levels of the studied genes were calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak, and Schmittgen 2001). The specific primers used for each gene are listed in Table 1.

Table 1. Primer sequences and product sizes used for quantitative RT-PCR.

Gene	Primer sequence (5'-3')	Size (bp)	Reference
Runx2	F: CACTGGCGCTGCAACAAGA R: CATTCCGGAGCTCAGCAGAATAA	127	(Qian et al. 2010)
OPN	F: ACCTGAACGCGCCTTCTG R: CATCCAGCTGACTCGTTTCATAA	66	(Dyson et al. 2007)
OC	F: CAAAGGTGCAGCCTTTGTGTC R: TCACAGTCCGGATTGAGCTCA	150	(Garlet et al. 2007)
BSP	F: CTGGCACAGGGTATACAGGGTTAG R: ACTGGTGCCGTTTATGCCTTG	182	(Fujii et al. 2008)
ALP	F: GGACCATTCCCACGTCTTCAC R: CCTTGTAGCCAGGCCCATTG	137	(Tomokiyo et al. 2008)
GAPDH	F: TCCTGCACCACCAACTGCTT R: TGGCAGTGATGGCATGGAC	100	(Fujii et al. 2008)

Abbreviations: OPN, osteopontin; OC, osteocalcin; BSP, bone sialoprotein; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

Annealing procedures were performed at 60°C for all primers.

7. Combining EGCG and Macroporous Biphasic Calcium Phosphate (MBCP) for Transplantation

A synthetic bone graft material, macroporous biphasic calcium phosphate (MBCP; Biomatlante, Bretagne, France), combined with EGCG was prepared for *in vivo* transplantation. The EGCG was dissolved in 75 μl of pure ethanol and the solution was dropped onto 40 mg of MBCP particles and completely dried out under a sterile hood. MBCP particles containing the following doses of EGCG were prepared: 0, 0.1, 0.5, and 1.5 mg. To evaluate the release of EGCG from its combination with MBCP, MBCP combined with 0.5 mg of EGCG was placed in 100 μl of 0.1 M Tris buffer solution (pH 7.4; Welgen, Daegu, Korea) at 37°C for 10 days. The amount of EGCG released into the buffer solution was measured every day using a spectrophotometer (Benchmark Plus Microplate spectrophotometer, Bio-Rad Laboratories) at 360 nm. After the release of EGCG was confirmed (Fig. 1), *in vivo* transplantation procedures were performed as described below.

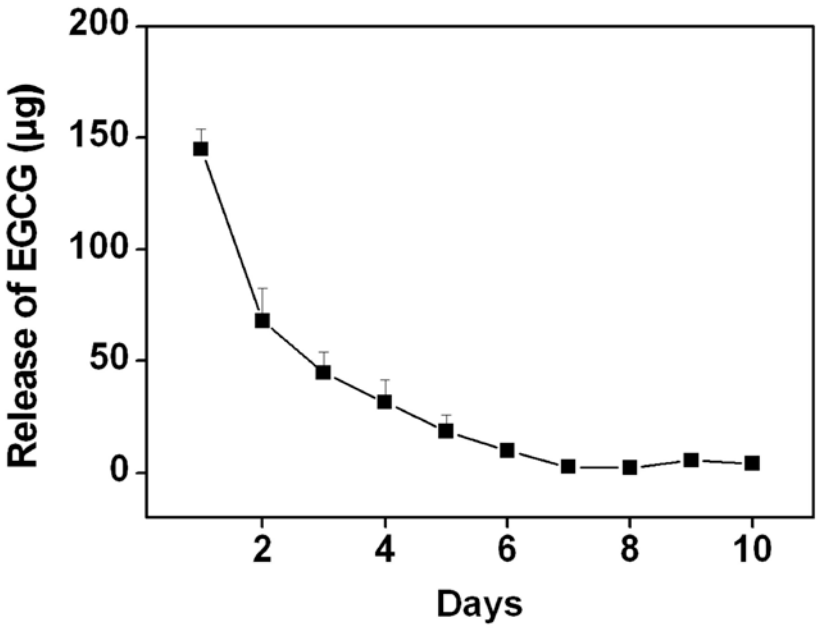


Figure 1. Daily release of (-)-epigallocatechin-3-gallate (EGCG) from macroporous biphasic calcium phosphate (MBCP) combined with 0.5 mg of EGCG. After 2 days approximately half of EGCG was released and there were little but consistantly releasing tendency through 10 days. The data were obtained from two independent experiments, with all samples run in duplicate. Data are mean ± standard deviation values.

8. *In vivo* Transplantation

The *in vivo* transplantation procedures were performed using a protocol approved by the Insititutional Animal Care and Use Committee of Yonsei University (#2011-0255). When the cells had expanded to a confluency of about 90% the expansion medium was changed to a medium containing 10 μM EGCG. After 24 h, *in vivo* transplantation procedures were performed as described previously (Kuznetsov et al. 1997). Approximately 3×10^6 cells were mixed with 40 mg of EGCG-combined MBCP and then incubated at 37°C for 2 h. The mixtures of cells and MBCP containing 0, 0.1, 0.5, and 1.5 mg of EGCG were transplanted into dorsal subcutaneous pockets (four pockets per mouse) in 5-week male immune-compromised mice (n=20; BALB/c-nu, SLC, Shizuoka, Japan). A mixture of EGCG-free MBCP and cells with no exposure to EGCG before transplantation was used as a control. In addition, MBCP particles without any cells were used as a negative control. Anesthetic fluid comprising Zoletil (Virbac, Carros, France), Rompun (Bayer, Leuverkeusen, Germany), and saline was injected into the right-lower intraperitoneal cavity of each mouse, and the combined experimental materials were then transplanted into the subcutaneous pocket on the back of the mouse. After 9 weeks, the mice were killed and all transplants were retrieved for further analysis.

9. Histological Analysis of the Transplants

The transplants (n=16 for each group) were fixed with 10% neutral-buffered formalin (Sigma) overnight and then decalcified with 10% EDTA (pH 7.4; Fisher

Scientific, Houston, TX, USA) for 3 weeks. Each transplant was divided into several fragments (about 1mm thickness), embedded in paraffin, and sectioned at a thickness of 3 μm. Sections were deparaffinized and then stained with hematoxylin and eosin (HE). The areas of newly formed hard tissue and of MBCP particles on each fragment of the HE-stained sections were measured using the Image J program (NIH), and the total area of the former was divided by the total area of the latter to evaluate the hard-tissue forming potential.

10. Gene expression analysis by quantitative RT-PCR of the transplants

The relative gene expressions of Runx2, BSP, OPN, and OC were evaluated by quantitative RT-PCR. Immediately after retrieval, the transplants (n=12 for each group) were immersed in RLT buffer (a component of the RNeasy Mini Kit, Qiagen), and homogenized by stainless-steel beads with a mean diameter of 0.5 mm (Next Advance, Averill Park, NY, USA) in a blender (Bullet, Next Advance). After centrifugation at 1×10^4 g with supernatant, quantitative RT-PCR was performed using the above-described procedure with the primers listed in Table 1. The expression levels of the gene were calculated relatively to their expression levels in the MBCP-only transplants.

11. Measurement of ALP Activity in the Transplants

The level of ALP activity in the retrieved transplants was measured using the Senso-Lyte® p-nitrophenylphosphate (pNPP) alkaline phosphatase assay kit (AnaSpec, Fremont,

CA, USA) according to the manufacturer's instructions. In brief, the retrieved transplants (n=8 for each group) were rinsed and soaked in PBS overnight. They were then lysed with TritonX-100 (provided in the kit), and *p*NPP was added to the supernatant of the lysates. The ALP activity was measured by the colorimetric change (absorbance at 405 nm) caused by dephosphorylation of *p*NPP. The level of ALP activity was normalized against the total amount of protein in the supernatant of the same tissue lysate using the Thermo Scientific Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

12. Statistical Analysis

Statistical analysis was performed with SPSS (version 19.0, SPSS, Chicago, IL, USA). Normality of the data was evaluated by the Shapiro-Wilk test (significance level of $p<0.05$). One-way ANOVA and the post-hoc Scheffé test (significance level of $p<0.05$) were applied to the data from the proliferation assay, cell migration assay, and Alizarin Red S staining. The Kruskal-Wallis test (significance level of $p<0.05$) followed by the Mann-Whitney U test (Bonferroni correction; significance level of $p<0.01$) were applied to analyze the results of HE staining analysis, quantitative RT-PCR, and ALP activity.

III. Result

1. Effects of EGCG on Proliferation, Apoptosis, and Cell Migration

The optical density, which reflected the total numbers of viable cells, tended to decrease as the EGCG concentration increased (Fig. 2), with this effect being significant for 50 μM EGCG. To evaluate whether the EGCG-mediated antiproliferative effect on hABCs was due to the induction of apoptosis, we performed flow cytometric analysis. As shown in Fig. 3, the number of apoptotic cells (subphase G0/G1) increased in the group treated with 50 μM (>25%), while it was lower than 8% in the other groups. The migration area was significantly smaller for 25 and 50 μM EGCG than for the other groups along the time passed (Fig. 4).

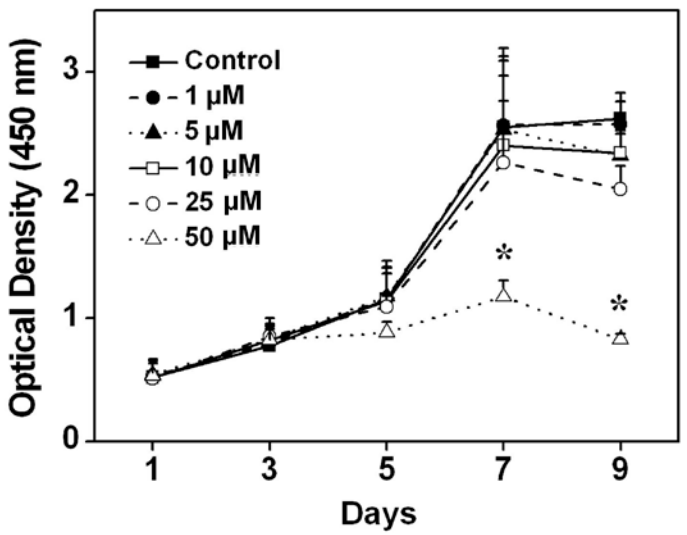


Figure 2. Effects of EGCG at various concentrations on the proliferation of human alveolar bone cells (hABCs). The *Y*-axis indicates the optical density of formazan converted by the cells. The data were obtained from three independent experiments, with all samples run in triplicate. Data are mean and standard deviation values. *Significantly different (at $p<0.05$) from all other groups in one-way ANOVA followed by the Scheffé test.

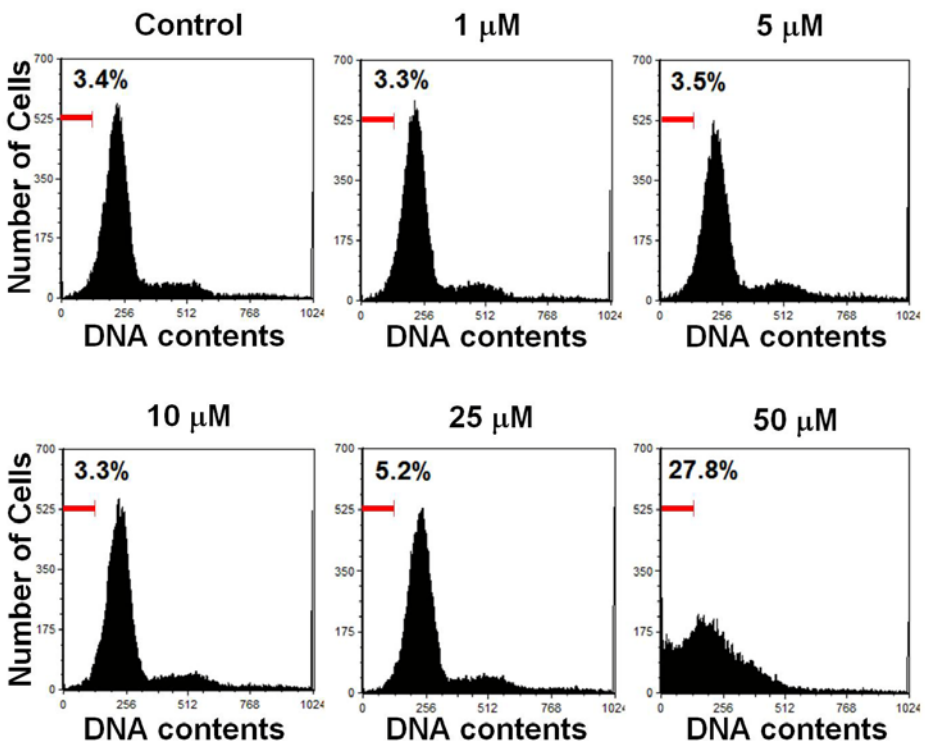
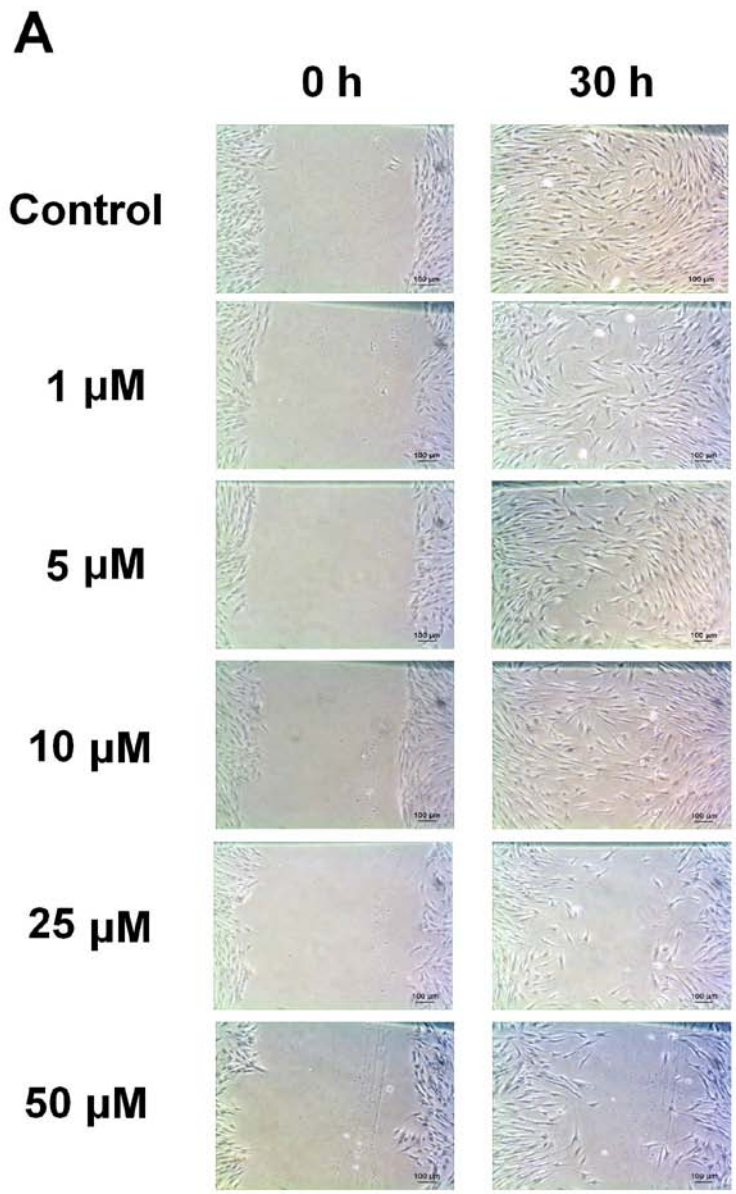


Figure 3. Effects of EGCG at various concentrations (0, 1, 5, 10, 25, 50 μM) on flow cytometric analysis for the apoptosis evaluation of hABCs. The number of apoptotic cells (subphase G0/G1) increased in the group treated with 50 μM (>25%). The apoptosis rate was higher for EGCG at 50 μM than at the other concentrations. The data were obtained from independent two separate experiments.


A
0 h
30 h
Control
1 μM
5 μM
10 μM
25 μM
50 μM

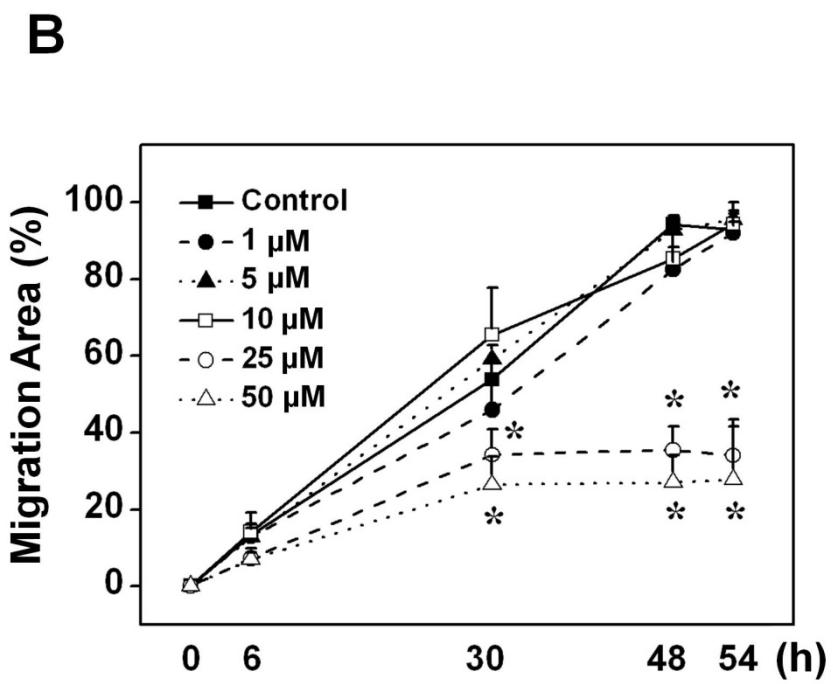
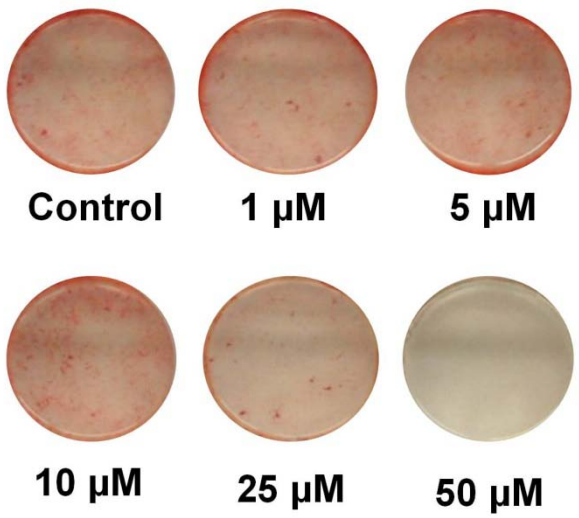


Figure 4. Effects of EGCG at various concentrations on cell migration. **(A)** Photographs of cell migration at 30 h (right) and immediately after wounding (left). Scale bars: 100 μm. **(B)** Cell migration area according to time and concentration of EGCG. The data were obtained from six independent experiments, with all samples run in triplicate. Data are mean and standard deviation values. *Significantly different (at $p<0.05$) in 25 and 50 μM EGCG group from the control group and the groups with 1, 5, and 10 in one-way ANOVA followed by the Scheffé test.

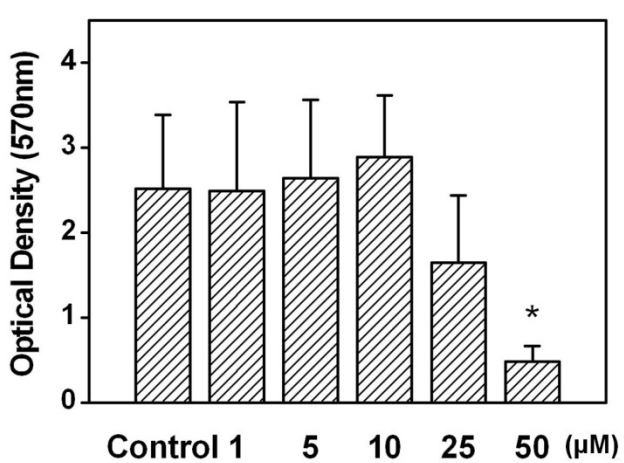
2. Effects of EGCG on *in vitro* osteogenic differentiation

Mineralization of the extracellular matrix increased slightly as the EGCG concentration increased up to 10 μM, but then decreased in the 25 and 50 μM groups, although the difference was significant only for 50 μM EGCG (Fig. 5A, B). When 10 μM EGCG (which showed the greatest increase in mineralization) was added to the osteogenic differentiation medium, the gene expression levels of ALP and BSP—which are related to the mineralization process—were higher than those of the control group at almost all time points (Fig. 5C). But there was no significant difference.

A


Control
1 μM
5 μM
10 μM
25 μM
50 μM

B


Optical Density (570nm)
0
1
2
3
4
*
Control 1
5
10
25
50 (μM)

C

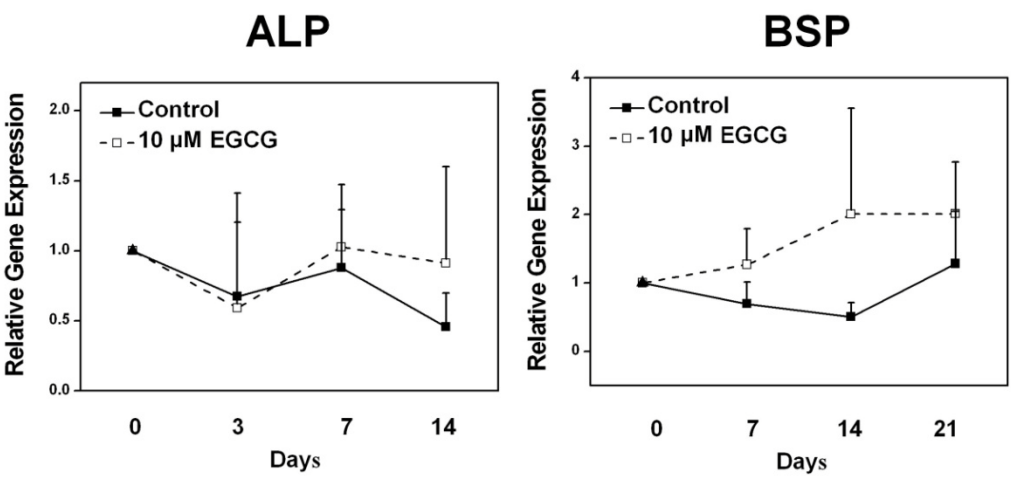
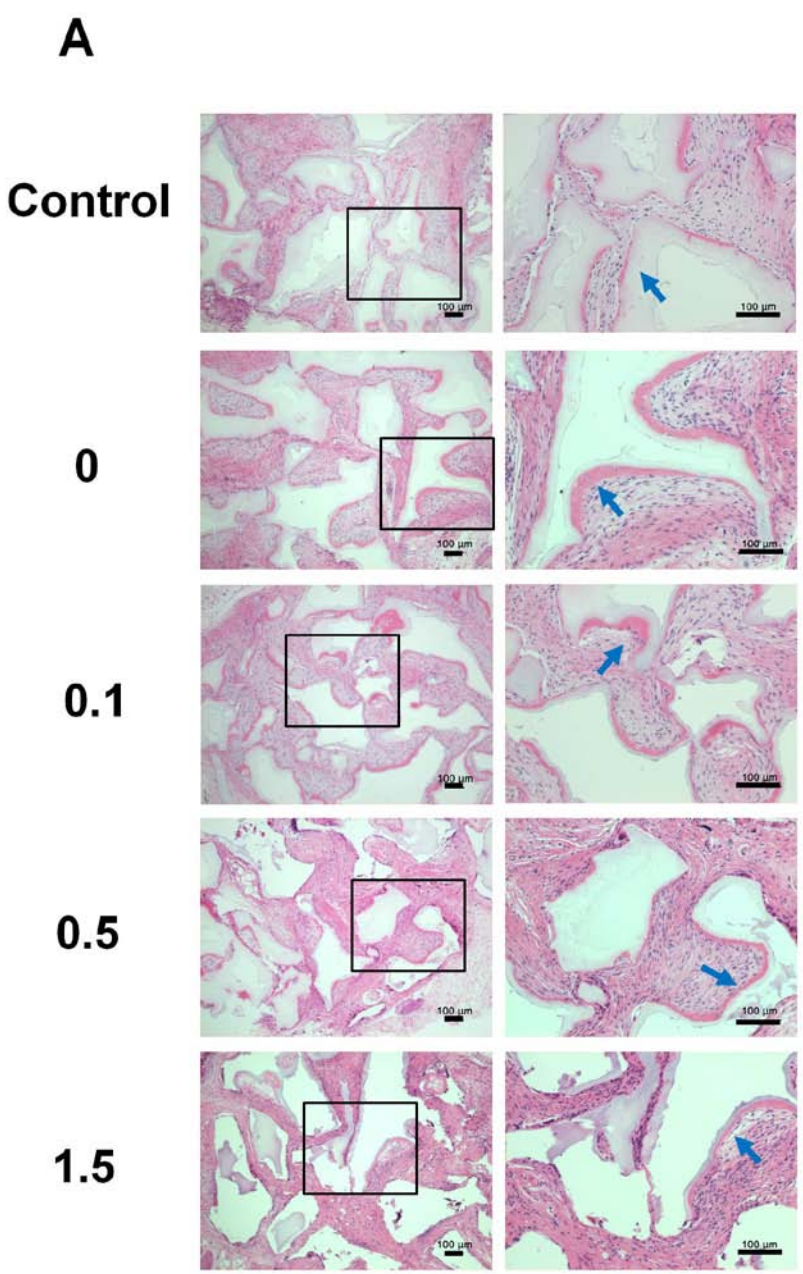


Figure 5. Effects of EGCG at various concentrations on the osteogenic differentiation of hABCs. **(A,B)** Alizarin Red S staining and its quantification. Dye accumulation data were obtained from six independent experiments, with all samples run in triplicate. Data are mean and standard deviation values. *Significantly different (at $p<0.05$) from control group in one-way ANOVA followed by the Scheffé test. **(C)** Changes in alkaline phosphatase (ALP) and bone sialoprotein (BSP) gene expressions during osteogenic differentiation in 10 μM EGCG and control groups. Gene expression data were obtained from six independent experiments, with all samples run in duplicate. Data are mean and standard deviation values.

3. Effects of EGCG on hard-tissue forming potential *in vivo*

As shown in Fig. 6, the amount of newly formed hard tissue was greatest in the group with MBCP and 0.1 mg of EGCG. The hard-tissue forming area was slightly smaller in the control group and the group with MBCP and no EGCG, but was markedly smaller in the groups with 0.5 and 1.5 mg of EGCG. In terms of RNA levels, the expressions of mineralization-related genes [early marker (Runx2) and late markers (OC, BSP, and OPN)] were highest in the group with 0.1 mg of EGCG, followed (in order) by the group with MBCP and no EGCG and the control group, and then they decreased sharply in the groups with 0.5 and 1.5 mg of EGCG (Fig. 7A). Finally, the level of ALP activity tended to increase slightly up to that for the group with 0.1 mg of EGCG, but then decreased significantly in the groups with 0.5 and 1.5 mg of EGCG (Fig. 7B).

Interestingly, the group with MBCP and no EGCG, in which the cells were incubated with 10 μM EGCG for 24 h prior to transplantation, exhibited greater hard-tissue forming potential than did the control group, in which the cells did not contact EGCG during the culture period prior to transplantation and MBCP was present without EGCG. In addition, although the cells were treated with EGCG prior to transplantation in the same manner, the potential for forming hard tissue was greater in the group with MBCP and 0.1 mg of EGCG than in the group with MBCP and no EGCG. However, this potential was significantly lower in the groups with more than 0.1 mg of EGCG per 40 mg of MBCP than in the control group.


A
Control
0
0.1
0.5
1.5
(EGCG, mg)
100 μm

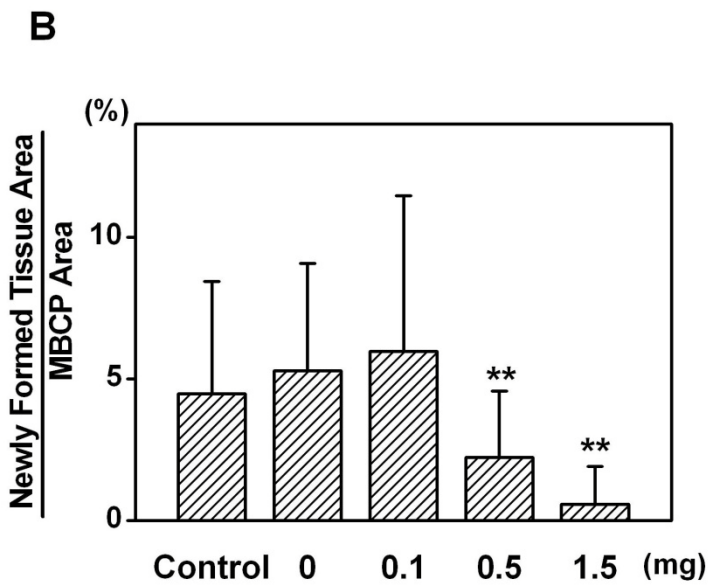


Figure 6. Hematoxylin and eosin (HE) staining and histological analysis in the transplants. **(A)** HE staining. The box area in the left is magnified to be shown in the right. Arrows indicate newly formed hard tissues adjacent to MBCP particles. Scale bars: 100 μm. **(B)** Histological analysis. The *Y*-axis indicates the percentage ratio of the newly formed tissue area relative to the MBCP particle area. Data are mean and standard deviation values. **Significantly different between the control, 0 and 0.1 mg of EGCG group and the groups with 0.5 and 1.5 mg of EGCG in the Kruskal-Wallis test (at $p<0.05$) followed by the Mann-Whitney U test (Bonferroni correction; at $p<0.01$).

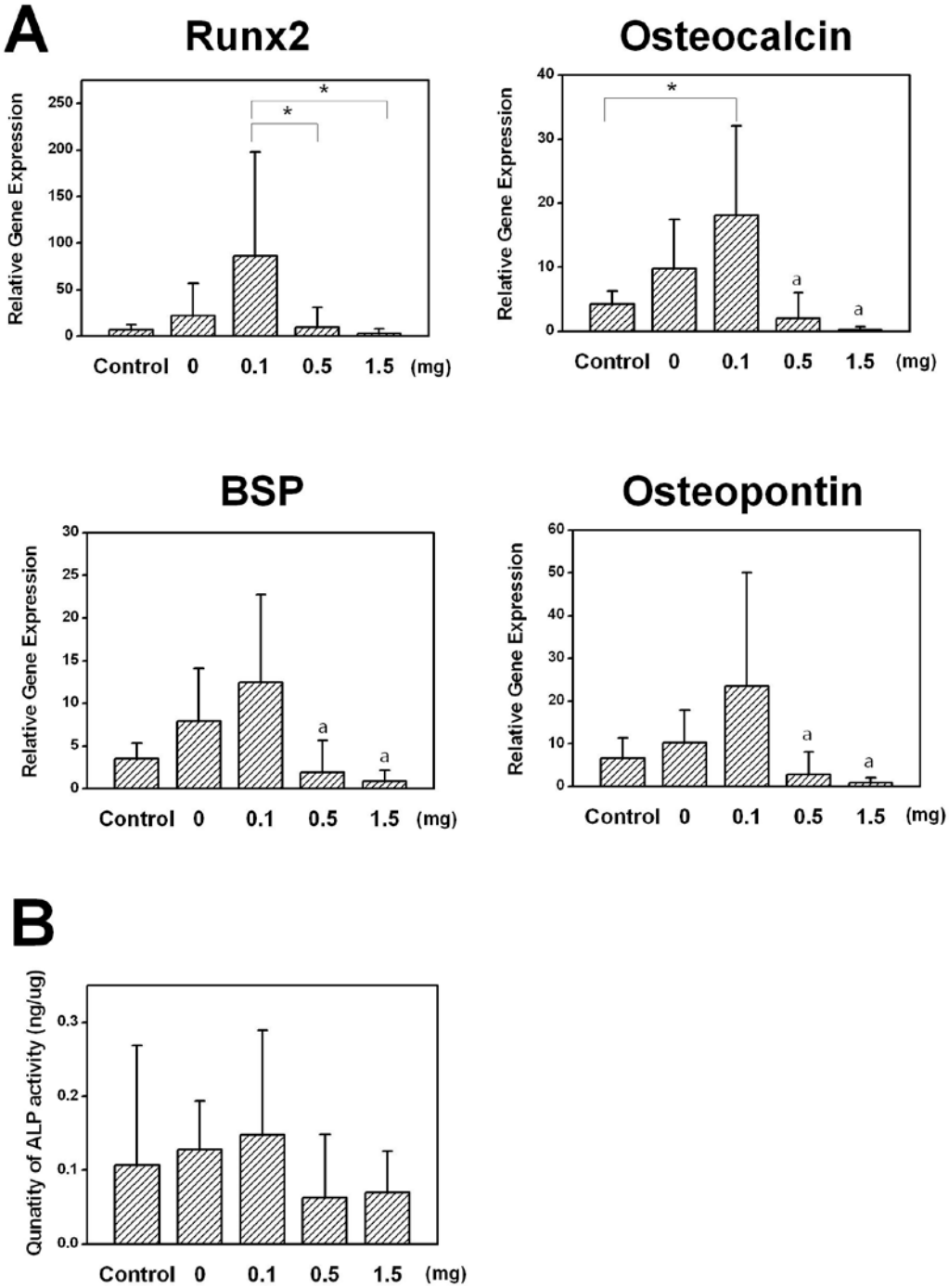

A
Runx2
Relative Gene Expression
250
200
150
100
50
0
*
*
Control 0 0.1 0.5 1.5 (mg)
Osteocalcin
Relative Gene Expression
40
30
20
10
0
*
a
a
Control 0 0.1 0.5 1.5 (mg)
BSP
Relative Gene Expression
30
25
20
15
10
5
0
a
a
Control 0 0.1 0.5 1.5 (mg)
Osteopontin
Relative Gene Expression
60
50
40
30
20
10
0
a
a
Control 0 0.1 0.5 1.5 (mg)
B
Qunatity of ALP activity (ng/ug)
0.3
0.2
0.1
0.0
Control 0 0.1 0.5 1.5 (mg)

Figure 7. Other gene expressions and levels of ALP activities in the transplants. **(A)** Relative gene expressions of Runx2, osteocalcin, BSP, and osteopontin. In the control group the cells were not pre-cultured with EGCG before transplantation, while in the '0' group they were pre-cultured with 10 μM EGCG for 24 h before transplantation, and no more EGCG was added with MBCP. The gene expression level of MBCP transplants was set as the control (normalized to 1). The data were obtained from 12 transplants for each group. Data are mean and standard deviation values. a: Significantly different between the control, 0 and 0.1 mg of EGCG group and the groups with 0.5 and 1.5 mg of EGCG. *Significantly different between each two groups in the Kruskal-Wallis test (at $p<0.05$) followed by the Mann-Whitney U test (Bonferroni correction; at $p<0.01$). **(B)** Relative ALP activities to the total proteins in the transplants. The data were obtained from eight transplants for each group. Data are mean ± standard deviation values.

IV. Discussion

There are various possible mechanisms through which EGCG in green tea could protect bone health, such as by mitigating bone loss through antioxidative stress activity (Itoh et al. 2005), anti-inflammatory activity (Hong et al. 2001, Tipoe et al. 2007), suppressing osteoclastogenesis (Nakagawa et al. 2002, Yun et al. 2007, Yun et al. 2004), and probably also osteoimmunological activity (Sato, and Takayanagi 2006, Shen et al. 2009, Wu et al. 2009). However, the current controversy about the effects on the osteoblast function prompted us to investigate this in the present study.

Several studies have found that EGCG exerted positive effects on osteoblast cells *in vitro*. Vali et al. (Vali, Rao, and El-Sohemy 2007) found that adding EGCG (at 1–5 μM) to human SaOS-2 cells (an osteosarcomal cell line) increased the ALP activity level and the numbers of mineralized nodules, suggesting that EGCG can induce the differentiation of osteoblasts to progress to the maturation level (Vali, Rao, and El-Sohemy 2007). Chen et al. (Chen et al. 2005) reported that EGCG at 1 and 10 μM increased the potential of osteoblast differentiation in D1 (murine bone marrow mesenchymal stem cells) by increasing ALP activity, and eventually stimulated mineralization. Our data indicated that treating osteoblasts with EGCG (at 1–10 μM) slightly increased the tendency for the formation of mineralized matrix and elevated the expressions of ALP and BSP genes. Increasing ALP is a direct indicator of osteoblastogenesis, and this increased mineralization, which is therefore also an indirect indicator of osteoblastogenesis.

On the other hand, negative effects of EGCG on osteoblast activity *in vitro* have also been reported. It was reported that EGCG (at 1–10 μM) suppressed the expressions of mRNA of ALP and OC and reduced the osteogenic differentiation of a precursor cell line of osteoblasts, MC3T3-E1, which was originally derived from mouse calvaria (Kamon, Zhao, and Sakamoto 2010). EGCG had no effect on ALP activity or mineralization in the differentiated MC3T3-E1 cells. Our results also showed the statistically significant suppression of osteogenic differentiation by high concentrations of EGCG (25 and 50 μM).

The present study showed that EGCG could both enhance and inhibit the osteogenic differentiation of hABCs *in vitro*, depending on the concentration of EGCG. Lower concentrations of EGCG (1, 5, and 10 μM) had slightly positive effects on osteogenic differentiation in hABCs, whereas higher concentrations could have clear negative effects, with 25 and 50 μM EGCG decreasing not only osteogenic differentiation but also the cell proliferation and migration abilities. The differences between the present study and previous studies could be due to differences in the experimental conditions, such as the use of different species (mouse or human) and cell types (primary-cultured cells or cell lines). However, the results of this study could be more applicable to the clinical context because we used normal human osteoblast cells and primary-cultured cells that did not have modified genomes (unlike when using cell lines).

Contradictory results have been reported for the *in vivo* effects of EGCG on osteoblasts. A recent study in which EGCG was combined at different doses (0, 0.1, 0.2, and 0.4 mg) with osteoconductive alpha-TCP particles (14 mg) and applied to rat calvarial bone

defects found that the induced bone regeneration was maximal for 0.2 mg of EGCG (Rodriguez et al. 2011). However, Takita et al. (Takita et al. 2002) showed that fibrous glass membrane mixed with rhBMP-2 (1.2 μg) and EGCG (at 1 or 10 μg) transplanted in rats increased cartilage formation and decreased bone formation. In both our *in vitro* and *in vivo* experiments, the formation of hard tissue varied with the amount of EGCG. The effectiveness was greater when using the low concentration in the group with 0.1 mg of EGCG, with EGCG levels exceeding 0.5 mg significantly reducing hard-tissue formation. This study is the first to have investigated the effects of preculturing in EGCG-containing culture medium before *in vivo* transplantation. The hard-tissue forming potential was greater when pretreating osteoblasts with 10μM EGCG than in the not-pretreated group. However, using a small amount (0.1 mg) of EGCG was effective than pretreatment because the MBCP scaffold that combined with EGCG could provide EGCG continuously to the osteoblasts. However, a limitation of this study could be the exclusion of other effects on the bone formation process *in vivo*. We used an ectopic model rather than a bone model *in vivo*. We focused on the new bone formation, with other factors such as osteoclastogenesis and angiogenesis not being evaluated, and hence they cannot be excluded.

The effects of low-dose EGCG in *in vivo* experiments might be due to the prevention of cell apoptosis, increased proliferation by stimulation of growth factor release from the osteoblasts, and direct enhancement of osteogenic differentiation. EGCG positively influences growth factors and antiapoptosis proteins (Aggarwal, and Shishodia 2006, Singh, Shankar, and Srivastava 2011). In addition, EGCG is also able to induce enzymes

that play important roles in cellular antioxidant defense mechanisms, such as glutathione peroxidase, and to scavenge the reactive oxygen species (Dreher et al. 1998, Nagai et al. 2002, Stangl et al. 2007) that might be involved in degrading the bone matrix and inhibiting differentiation of osteoblasts, resulting in promotion of bone resorption and inhibition of bone formation (Suzuki et al. 1997). Transplanting the cell and scaffold mixtures into the *in vivo* environment in the subcutaneous pockets of the nude mice would induce ischemic, mechanical, and nutritional stresses. However, EGCG would help the osteoblasts to survive in this difficult environment.

Meanwhile, another study has found that EGCG at low concentrations had a protective effect on DNA, whereas at higher concentrations it enhanced DNA oxidative damage (Tian et al. 2007) -at low concentrations, ROS scavenging activity of EGCG might predominate over its reducing power and lead to its protective effect on DNA, however, the higher reducing power of EGCG at higher concentrations may gradually predominate over its ROS scavenging activity and result in the prooxidant effect of EGCG on DNA (Tian et al. 2007). In addition, EGCG has an effect on phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling which is involved in multiple cell functions including cell proliferation, apoptosis, and differentiation in a dose-dependent manner (Natsume et al. 2009). At low concentration of EGCG, up-regulating PI3K/AKT signaling greatly enhanced DNA binding of Runx2 resulting in Runx2-induced osteoblastogenesis (Fujita et al. 2004). However, EGCG at high concentration significantly suppressed phosphorylation of the PI3K/AKT pathway leading to cell death (Natsume et al. 2009, Zhang et al. 2007). This is consistent with the higher levels of

EGCG inhibiting proliferation, migration, and osteogenic differentiation in both the *in vitro* and *in vivo* experiments performed in the present study.

V. Conclusion

In conclusion, this study has shown that EGCG at higher concentrations can exert negative effects on the osteogenic differentiation of hABCs both *in vitro* and *in vivo*, while at appropriate low concentrations EGCG might slightly enhance the osteogenic effect with pre-incubation *in vitro*. EGCG will exert either positive or negative effects on the same osteoblast cells, depending on its concentration. In other words, the clinical application of EGCG at an appropriate concentration could encourage osteoblast activity, while at higher concentrations it should suppress osteoblasts.

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국문요약

녹차추출물(Epigallocatechin-3-gallate)의 사람 치조골모세포에 대한 골분화 유도 효과

연세대학교 대학원 치의학과
마 연 주
지도교수: 최형준

녹차의 성분 중 많은 양을 차치하는 EGCG의 현재 알려진 다양한 효능 중에서 골의 대사와 관련하여 사람과 쥐의 세포를 대상으로 한 연구 결과가 서로 상반된 osteoblastogenesis 에 대한 영향을 보고하였으며 대부분의 연구는 사람과 쥐의 암세포를 대상으로 한 실험으로 진행되었다. 이번 연구에서는 EGCG 가 사람의 정상 치조골 세포에서 유래된 조골세포에 대해 *in vitro, in vivo* 상에서의 직접적인 골형성능에 대한 영향을 평가하고자 하였다.

사람의 치조골로부터 일차배양한 골모세포(hABCs)를 준비하여 다양한 농도의 EGCG (0, 1, 5, 10, 25, 50 μM) 를 적용하였다. 이 세포를 가지고 *in vitro* 상 증식, 세포사에 대한 유세포분석, 세포이동능 분석을 진행하였고, *in vivo* 상 10 μM EGCG를 이식하기 하루 전 적용시킨 hABCs 을 0, 0.1, 0.5, 1.5 mg의 EGCG와 macroporous biphasic calcium phosphate(MBCP)에 혼

합하여 쥐에 이식한 후 9주 경과한 시점에서 다시 채취하여 조직학적 분석, 정량적 역전사 중합효소 연쇄반응 분석, ALP 활성도 측정을 시행하였다.

In vitro 에서 EGCG 를 고농도로 처리한 경우(25, 50 μM) 골모세포의 증식과 이동능이 확연히 억제되는 결과를 보였고, 10 μM 이하에서는 농도가 증가함에 따라 증식이 미미한 정도로 증가하는 경향을 보였으며 분화와 관련하여 10 μM 의 EGCG 를 처리하였을 때 가장 증가된 광화작용을 보였다. *In vivo* 에서 0.1 mg 의 EGCG 를 처리한 경우 경조직 형성능이 높게 나타났고 그 이상의 고농도로 적용되는 경우에는 유의성 있게 낮은 결과를 보였다.

EGCG 를 적정한 농도로 적용한 경우 hABCs의 골형성에 다소 긍정적인 영향을 줄 수 있으나 *in vitro*, *in vivo* 의 두 가지 경우 모두 EGCG 가 고농도로 적용될 시에는 골형성에 대한 효과가 확연히 억제된다.

핵심되는 말: 치조골유래세포, EGCG, 골분화, 생체내 이식,