BIOCHEMICAL PHARMACOLOGY 72 (2006) 1268–1278

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Quercetin, a flavonoid, inhibits proliferation and increases osteogenic differentiation in human adipose stromal cells

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ARTICLE INFO

Article history:
Received 8 June 2006
Accepted 22 August 2006

Keywords:
Quercetin
Osteogenic differentiation
Human adipose stromal cells
Proliferation
ERK
Bone regeneration

ABSTRACT

Flavonoids, which have been detected in a variety of foods, have been repeatedly reported to affect bone metabolism. However, the effects of flavonoids on osteoblastogenesis remain a matter of some controversy. In this study, the effects of quercetin on the differentiation and proliferation of human adipose tissue-derived stromal cells (hADSC) were determined. Quercetin was found to increase osteogenic differentiation in a dose-dependent manner. Other flavonoids, chrysin and kaempferol, were also shown to increase the osteogenic differentiation of hADSC, but this stimulatory effect was weaker than that associated with quercetin. Quercetin pretreatment administered prior to the induction of differentiation also exerted stimulatory effects on the osteogenic differentiation of hADSC. RT-PCR and real time PCR analysis showed that quercetin treatment induced an increase in the expression of osteopontin, BMP2, alkaline phosphatase and Runx2. Quercetin inhibited the proliferation of hADSC, but did not affect their survival. The pretreatment of quercetin increased ERK phosphorylation during osteogenic differentiation, although it did not increase ERK activity in control culture condition. ICI182780, an specific estrogen receptor antagonist, failed to inhibit the effects of quercetin on osteogenic differentiation. Quercetin-pretreated hADSC showed better bone regenerating ability in skull defect model of nude mice than naive cells. Our findings indicate that quercetin enhances osteogenic differentiation via an independent mechanism from estrogen receptor (ER) activation, and prove useful for *in vivo* bone engineering, using human mesencymal stem cells (hMSC).

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1. Introduction

Mesenchymal stem cells (MSC), which can be isolated from bone marrow, adipose tissue, and cord blood, are defined as pluripotent cells with limited self-renewal capacity, and can be differentiated into four classes: the adipocytes, chondrocytes, myoblasts, and osteoblasts [1–4]. Therefore, MSC constitute an interesting target for use in gene therapy [5] and cell therapy, and provide a unique model for the better understanding of early differentiation events, as they are able to differentiate into multiple mesenchymal lineages.

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doi:10.1016/j.bcp.2006.08.021

Conventional therapies, including autogenous bone grafts, allograft implants, and prosthetic implants, have been classically employed in the treatment of these problems. However, these methods are limited, due both to issues of supply and of osteogenic potential. Recent advances in cell and molecular biology have enabled researchers in the bone tissue engineering field to use cell and gene therapies. Stem cell therapy using adult-derived stem cells, particularly MSC, is an emerging field with regard to bone regeneration. The genetic modification of MSC and the addition of BMP2 have both been studied in attempts to improve the *in vivo* bone regeneration ability of MSC [6]. However, genetic modification protocols are associated with certain safety issues in clinical applications. Therefore, the screening of chemicals that enhance osteogenic differentiation may serve to expand the clinical applications of bone engineering using MSC.

A variety of studies have begun to amass evidenced regarding the neuroprotective, cardioprotective, and chemopreventive actions of the dietary flavonoids. Whereas the primary focus has been placed on the antioxidant properties of these flavonoids, there is an emerging view that flavonoids, as well as their *in vivo* metabolites, do not function as conventional hydrogen-donating antioxidants, but may instead exert modulatory actions in cells via their actions at the protein kinase and lipid kinase signaling pathways. Flavonoids, and more recently their metabolites, have been reported to function at the phosphoinositide 3-kinase (PI 3-kinase), Akt/protein kinase B (Akt/PKB), tyrosine kinases, protein kinase C (PKC), and mitogen activated protein kinase (MAP kinase) signaling cascades. Inhibitory or stimulatory effects at these pathways are likely to modulate cellular functions profoundly, via alterations of the phosphorylation states of target molecules, and via the modulation of gene expression.

Isoflavones, including daidzein and genistein, which are detected abundantly in soybeans and their derivative foods, have been determined to reduce the occurrence of osteoporosis in a variety of experimental models [7,8], as well as in a recent clinical study [9]. Moreover, the low reported incidence of osteoporosis in Asian women has been attributed to an isoflavone-rich diet [10,11]. The hypothesis that flavonols might also be bioactive molecules, which may be able to counteract the deleterious effects of estrogen deficiency occurring during menopause, has been recently addressed by Horcajada-Molteni et al. [12], who demonstrated that rutin, a glycoside derivative of quercetin, one of the major flavonols, inhibits ovariectomy-induced osteopenia in female rats. Quercetin is one of the principal flavonoids detected in certain plant species (for example, 200–600 mg quercetin/kg onion), in which it exists primarily in the form of glycosides (rutin) [13]. Dietary glycosides are converted to aglycones (such as quercetin) in the large intestine, in reactions catalyzed by the glycosidases generated by intestinal bacteria [14].

It has recently been demonstrated that flavonols such as quercetin and kaempferol effect a reduction in osteoclastic bone resorption *in vitro* via the direct targeting of the mature osteoclasts by a mechanism involving, at least in part, the estrogen receptor (ER) [15]. However, the role of quercetin in osteoblastogenesis remains controversial. Notoya et al. [16] reported that quercetin inhibits the proliferation, differentiation, and mineralization of rat calvarial osteoblast-like cells *in vitro*, whereas it inhibits osteoclastic activity [17]. However, Prouillet et al. [18] reported that quercetin and kaempferol induced an increase in alkaline phosphatase activity in MG-63 human osteoblasts via the activation of the estrogen receptor, and Miyake et al. [19] reported on the promoting effect of kaempferol on the differentiation and mineralization of a murine pre-osteoblatic cell line.

Human adipose tissues also possess multipotent progenitor cells, which can differentiate into osteoblasts [20]. Therefore, human adipose tissue-derived stromal cells (hADSC) constitute a good source for bone tissue engineering, due to the abundance of adipose tissues in the human body. In the previous study, we reported that pretreatment with valproic acid effected an increase in osteogenic differentiation in hADSC [21].

In this study, we assessed the effects of flavanols on the osteogenic differentiation of hADSC, and the relevant action mechanisms. Our results showed that quercetin pretreatment resulted in an increase in osteogenic differentiation in hADSC and bone regeneration *in vivo*, and that the effect on osteogenic differentiation was not associated with the estrogenic action of quercetin.

2. Materials and methods

2.1. Materials

Quercetin, kaempferol, chrysin, 17-β-estradiol (Sigma, USA), and ICI182780 (Tocris bioscience, USA) were dissolved in dimethylsulfoxide (DMSO), and applied as indicated in the figure legends. The levels of DMSO were maintained at under 0.1%.

2.2. Culture of MSC

All protocols involving human subjects were approved by the Institutional Review Board of the Pusan National University [22]. hADSC were isolated in accordance with the methods described in the previous studies. In order to isolate the hADSC, the adipose tissues were washed in phosphate-buffered saline (PBS), and the tissues were digested for 30 min at 37 °C with 0.075% type I collagenase. Enzyme activity was neutralized using α-modified Eagle's medium (α-MEM), containing 10% fetal bovine serum (FBS), and centrifuged for 10 min at 1200 × *g* to obtain a pellet. This pellet was then incubated overnight at 37 °C/5% CO_2 in control medium (α-MEM, 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin). After incubation, the tissue culture plates were washed in order to remove any residual nonadherent cells, then maintained at 37 °C/5% CO_2 in control medium. When the monolayer of adherent cells had achieved confluence (P0), the cells were trypsinized (0.25% trypsin; Sigma, USA), resuspended in α-MEM containing 10% FBS, and subcultured at a concentration of 2000 cells/cm^2. For most of the experiments, we used the hADSC at the 3rd–5th passages.

2.3. Induction of differentiation

Osteogenic differentiation was induced via the culturing of the cells for a minimum of 2 weeks in osteogenic medium (OM,

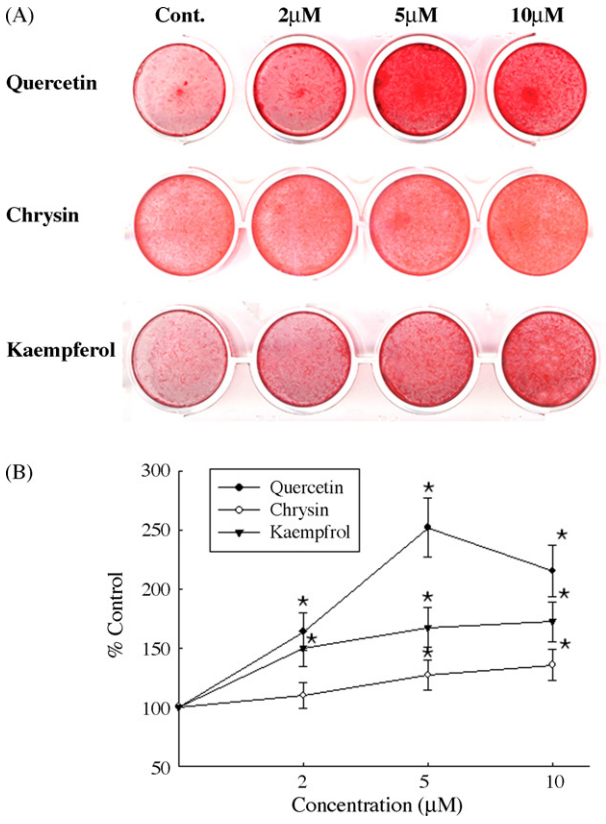

Fig. 1 – Effects of flavonoids on osteogenic differentiation of hADSC. The treatment of flavonols during the first 3 days of differentiation resulted in a significant increase in osteogenic differentiation in hADSC. (A) hADSC were treated with various concentrations (2–10 μM) of quercetin, chrycin, and kaempferol during the initial phase of osteogenic differentiation (the first 3 days). Osteogenic differentiation was determined by alizarin red S staining of calcification deposits within the cell monolayer. (B) The quantitation of osteogenic differentiation was performed by determination of density and area of alizarin red S staining with an image analysis program (Multi Gauge V3.0, FUJIFILM). Data were presented as a percentage of control (mean ± S.E.M., $n = 3$). $^{*}P < 0.05$ compared with control (cont.).

10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μM ascorbic acid in α-MEM), and extracellular matrix calcification was estimated using Alizarin red S stain. Osteogenic differentiation was quantified via the measurement of the alizarin red-stained area and density in 12-well dishes, using an image analysis program (Image Gauge ver 3.1, Fuji, Japan). Measurements were conducted in duplicate in each of the experiments, and the experiments were repeated with samples from at least three different donors.

2.4. *Reverse-transcription-polymerase chain reaction (RT-PCR) analysis*

Total cellular RNA was isolated from hADSC, and reverse transcribed using the conventional protocols. PCR amplification was conducted using the following primer sets: GAP-DH 5′-TCCATGACAACTTTGGTATCG-3′, 5′-TGTAGCCAAATTCGTTGTCA-3′, BMP2 5′-CCACCATGAAGAATCTTTGG-3′, 5′-CCACGTACAAAGGGTGTCTC-3′, alkaline phosphatase 5′-TGAAATATGCCCTGGAGC-3′, 5′-TCACGTTGTTCCTGTTTAG-3′. All primer sequences were determined using the established GenBank sequences. Duplicate PCR reactions were amplified with primers designed with GAPDH as a control for the evaluation of PCR efficiency and for subsequent analysis via agarose gel electrophoresis.

2.5. *Real time polymerase chain reaction (PCR)*

cDNA was synthesized in a reaction containing 1 μg of DNase I-treated total RNA of control hADSC and quercetin-treated hADSC, oligo dT primer, dNTP, and avian myeloblastosis virus (AMV) reverse transcriptase. Primer sequences to be used in the experiment were as follows: β-actin, 5′-CTG GTG CCT GGG GCG-3′, 5′-AGC CTC GCC TTT GCC GA-3′; Runx2, 5′-CTCACTACCACACCTACCTG-3′, 5′-TCAATATGGTCGCCAAACAGATTC-3′, osteopontin 5′-TTGCAGTGATTTGCTTTTGC-3′, 5′-ACACTATCACCTCGGCCATC-3′, BMP2 5′-CCACCATGAAGAATCTTTGG-3′, 5′-CCACGTACAAAGGGTGTCTC-3′.

Real time quantitation was predicated on the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture for PCR with the LightCycler Instrument (Roche, Germany). All experiments were conducted three times, and both negative and positive controls were included. Whereas no template

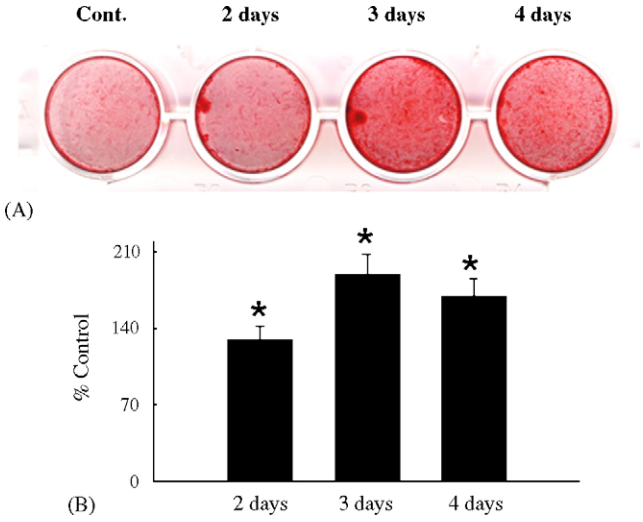

Fig. 2 – Effect of quercetin pretreatment on the osteogenic differentiation of hADSC. Quercentin pretreatment increased osteogenic differentiation of hADSC. (A) hADSC were pretreated with 5 μM quercetin for the indicated days before induction of osteogenic differentiation. Osteogenic differentiation was determined by alizarin red S staining of calcification deposits within the cell monolayer. (B) The quantitation of osteogenic differentiation was performed by determination of density and area of alizarin red S staining with an image analysis program (Multi Gauge V3.0, FUJIFILM). Data were presented as a percentage of control (mean ± S.E.M., $n = 3$). $^{*}P < 0.05$ compared with control (cont.).

negative control (H_2O control) was run with every gene specific primer, the no RT-PCR control was run with only one primer pair, which could amplify contaminated genomic DNA. Statistical significance was determined via ANOVA. For each of the primer pairs, the linearity of detection was verified to have a correlation coefficient of at least 0.98 over the detection area, via the measurement of a dilution curve with the cDNA isolated from the hADSC. β-Actin mRNA was amplified as an internal control. We used the comparative C_t method to quantify changes in gene expression between control and quercetin-treated hADSC where C_t denotes the cycle at which fluorescence above background levels becomes significant. The fold-difference between control and quercetin-treated samples is calculated according to $2^{-\Delta\Delta C_t}$. For each control and quercetin-treated sample, ΔC_t represents the difference between C_t of a target gene and the internal control (i.e., β-actin). The $\Delta\Delta C_t$ value represents the difference between ΔC_t values for a control sample and quercetin-treated sample.

2.6. Western blot analysis

Confluent hADSCs were treated under the appropriate conditions and lysed, after which their protein contents were determined using a protein assay kit (Bio-Rad Laboratories, USA). The proteins were loaded on 10% SDS polyacrylamide gels, electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech, USA), and probed with monoclonal antibodies (antiphospho-ERK (Thr202/204), anti-ERK antibody; Cell Signaling Technology, USA). Immunoreactive bands were detected with anti-rabbit peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, USA) and visualized via enhanced chemiluminescence (ECL detection kit, Amersham Pharmacia Biotech, USA).

2.7. Evaluation of cell proliferation and viability

In order to determine the proliferation rate, the cells were detached using Hank's balanced salt solution (HBSS) containing 0.5% trypsin and 0.02% EDTA. The hADSC were plated at a density of 1×10^4 cells/well in 12-well plates in the presence or absence of various concentrations of quercetin. After 1st, 2nd and 3rd day, the cells were trypsinized and stained with 0.4% trypan blue (Sigma, St. Louis, MO). The total cell number and the proportion of dead cells were measured with a hemocytometer. Cell death was determined by the presence of cytoplasmic trypan blue. This experiment was performed in triplicate.

2.8. In vivo implantation and harvesting

Scaffolds (collagen matrix; B. Braun Melsungen AG, Germany) were cut into 4 mm-diameter disks, then seeded with 1×10^6 cells. Cells in a 50 μl volume were pipetted several times into α-MEM. The bone formation of the quercetin-pretreated-hADSC was evaluated in a calvarial defect-induced

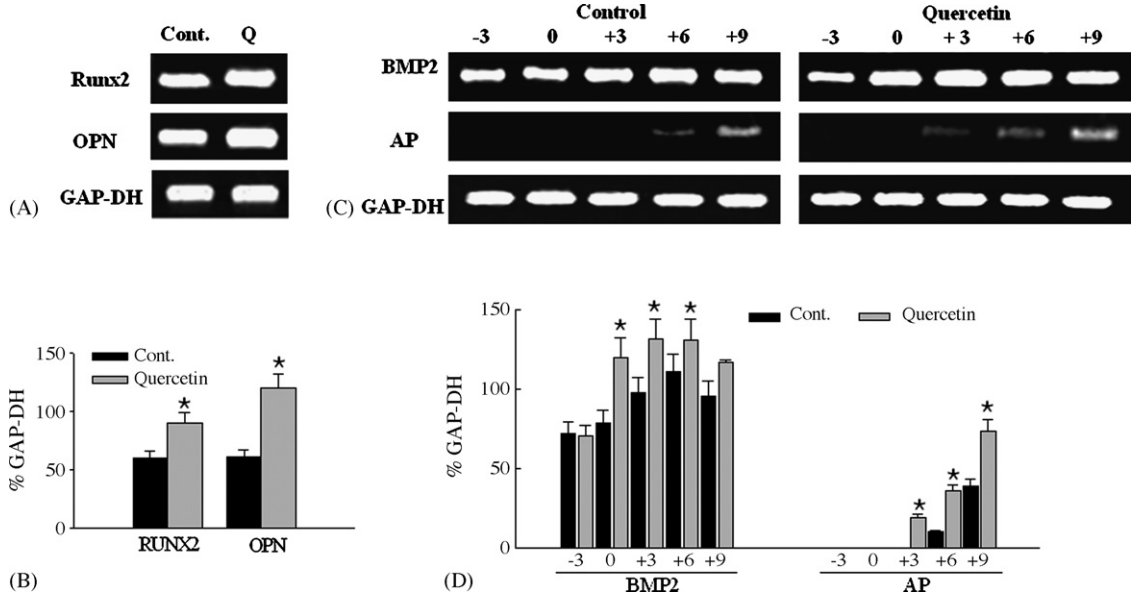

Fig. 3 – RT-PCR anaylsis of osteogenic genes. Quercetin pretreatment increased expression of osteogenesis-related genes. Control hADSC or hADSC pretreated with 5 μM quercetin for 3 days were induced for up to 9 days in osteogenic medium and analyzed by RT-PCR for the indicated genes. (A) Changes in expression of Runx2 and osteopontin (OPN) genes after treatment of quercetin in hADSC. Total RNA were isolated from control hADSC (cont.) or from hADSC after 3 days pretreatment of quercetin (Q). (C) Changes in expression of BMP2 and alkaline phosphatase (AP) genes during osteogenic differentiation of control or quercetin-pretreated hADSC. Total RNA were isolated from hADSC before plating (−3), from hADSC grown for 3 days in control or quercetin-containing media before induction of differentiation (0), and from control or quercetin-pretreated hADSC incubated in osteogenic media for 3 days (+3), 6 days (+6) and 9 days (+9). (B and D) Relative quantity of amplified products was analyzed by an image analyzer. Data represent mean ± S.E.M. of the relative ratio to GAPDH signal of the corresponding samples ($n = 3$). $^*P < 0.05$ compared with control (cont.).

murine model, using 8 week-old Balb/C nude mice (Sam:-TacN(SD)fbr). In order to induce the calvarial defects, the animals were anesthetized with pentobarbital sodium (0.5 mg/g). The surgical site was cleaned with ethanol, and an incision was cut just off the sagittal midline, exposing the parietal bone. A 4 mm craniotomy was conducted using a dental drill at slow speed, using a trephine burr. The drilling site was irrigated with saline and a lidocaine/epinephrine solution in order to limit superficial bleeding. The scaffold was set into the created cranial defect, then gently press-fitted into place around the edges. After the placement of the scaffold into the defect, the skin was sutured closed, and the animal was monitored. Samples were harvested 5 weeks after surgical implantation. The calvaria were fixed for 24 h in 10% formalin at 4 °C. Skulls were stained with 1 mg/ml alizarin red for 12 h in order to visualize bone formation. Mineralization of the defect was quantified as a function of the number of pixels within the area.

2.9. Histological evaluation

The skulls were fixed in 10% neutral buffered formalin or 4% paraformaldehyde, followed by decalcification in 10% EDTA. Paraffin-embedded samples were sectioned to a thickness of 10 μm with a microtome. The sections were then floated in a water bath at 40 °C, positioned on poly-L-lysine-coated microscope slides and baked overnight at 37 °C. For hematoxylin and eosin (H&E) staining, the sections were dewaxed in xylene and rehydrated in ethanol baths. The nuclei were stained with hematoxylin stain for 8 min and eosin for 10 dips. The sections were then covered with Permount and coverslipped.

2.10. Statistical analysis

All results are presented as the means ± S.E.M. Comparisons between groups were analyzed via *t*-tests (two-sided) or ANOVA for experiments with more than two subgroups. Post hoc range tests and pairwise multiple comparisons were conducted using the *t*-test (two-sided) with Bonferroni adjustments. Probability values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Flavonols increases osteogenic differentiation

In order to determine whether the flavonols affected osteogenic differentiation, the hADSC were treated with various concentrations (2–10 μM) of quercetin, chrysin and kaempferol during the initial phase of osteogenic differentiation (the first 3 days). The treatment of flavonols during the first 3 days of differentiation resulted in a significant increase in osteogenic differentiation in hADSC, and this occurred in a dose-dependent manner. When cells were treated with quercetin for more than 3 days in osteogenic media, cells detached from the culture dishes due to nonspecific cell damage (data not shown). The potency of flavonols for the induction of osteogenic differentiation was

Table 1 – C_t values for BMP2, Runx2 and OPN in control and Quercetin-treated hADSC

Samples	BMP2	Runx2	OPN	β-Actin	ΔC_t of BMP2	ΔC_t of Runx2	ΔC_t of OPN	$\Delta\Delta C_t$ of BMP2	$\Delta\Delta C_t$ of Runx2	$\Delta\Delta C_t$ of OPN	Fold of BMP2	Fold of Runx2	Fold of OPN
Control	24.11 ± 0.04	27.48 ± 0.05	28.2 ± 0.04	20.23 ± 0.04	3.88	7.25	7.64	0	0	0	1	1	1
Quercetin-treated	21.61 ± 0.04	26.33 ± 0.05	25.72 ± 0.05	20.06 ± 0.05	1.55	6.27	5.49	2.33	0.98	2.15	5.16	2.22	5.89

Values represent mean ± S.E.M. ($n = 3$). Control values are subtracted from Quercetin-treated values to obtain $\Delta\Delta C_t$ and fold-difference.

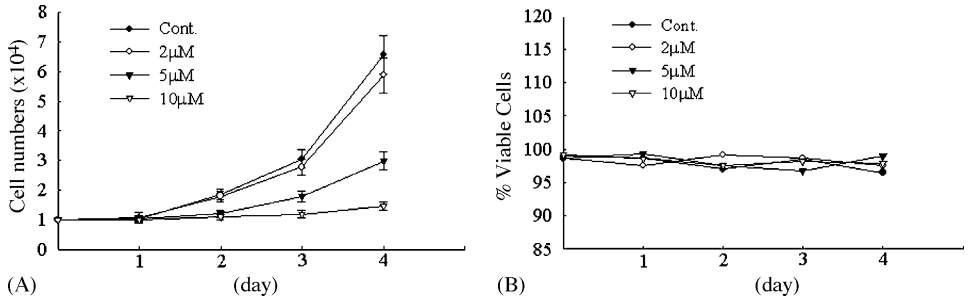

Fig. 4 – Effect of quercetin on the proliferation of hADSC. Quercetin inhibited proliferation of hADSC in a dose-dependent manner. The next day after plating hADSC (2000 cells/cm^2), various concentrations of quercetin were added. (A) Proliferation of hADSC was determined by direct cell counting using a hemocytometer at the 1st, 2nd and 3rd day after the addition of quercetin. (B) The percentage of dead cells was determined by trypan blue staining Data represent mean ± S.E.M. (n = 4). $^*P < 0.05$ compared with the data in the absence of quercetin.

as follows: quercetin > kaempferol > chrysin (Fig. 1). Quercetin did not affect adipogenic differentiation (data not shown).

We then attempted to determine whether pretreatment with quercetin would be sufficient for the enhancement of osteogenic differentiation. After quercetin pretreatment in control culture medium, the osteogenic differentiation of the hADSC was induced without the addition of quercetin (Fig. 2). hADSC pretreated with 5 μM quercetin showed increased calcification of extracellular matrix compared with control cells, and pretreatment of hADSC with quercetin for 3 days showed the greatest osteogenic differentiation Therefore, the MSC were pretreated with 5 μM quercetin for 3 days in the following experiments.

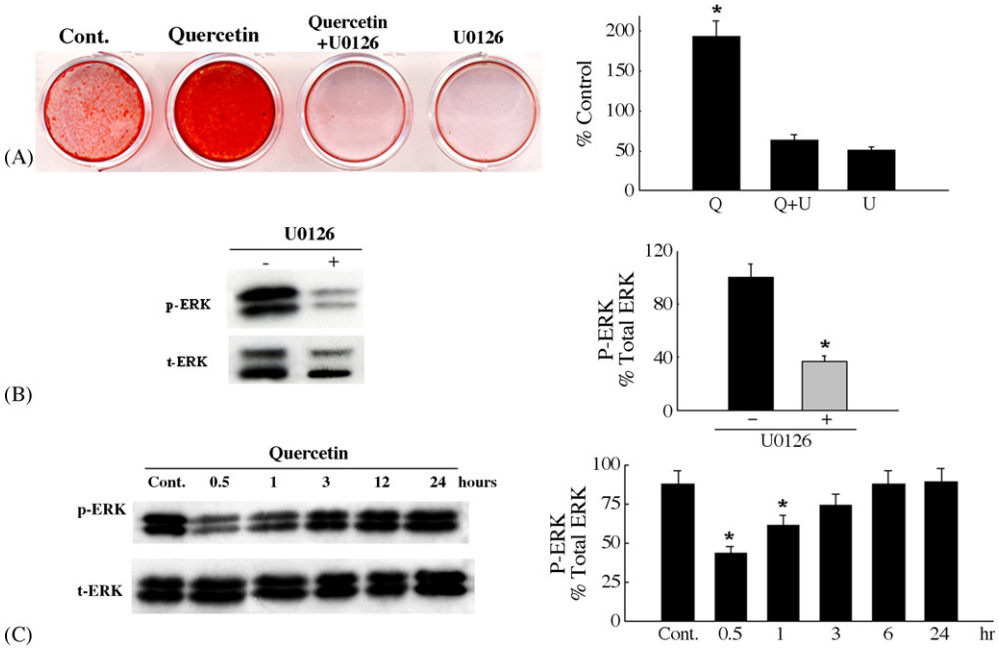

Fig. 5 – Effects of U0126 and quercetin on osteogenic differentiation and ERK activity of hADSC. An ERK inhibitor, U0126, inhibited quercetin-induced osteogenic differentiation in hADSC, but quercetin itself failed to increased ERK phosphorylation. (A) Control hADSC or hADSC pretreated with 5 μM quercetin (Q) were induced into osteogenic differentiation in the absence or presence of 10 μM U0126 (U) for 2 weeks. Osteogenic differentiation was determined by alizarin red S staining of calcification deposits within the cell monolayer. The quantitation of osteogenic differentiation was performed by determination of density and area of alizarin red S staining with an image analysis program (Multi Gauge V3.0, FUJIFILM). Data were presented as a percentage of naïve hADSC (mean ± S.E.M., n = 3). $^*P < 0.05$ compared with quercetin-nontreated hADSC in the presence or absence of U0126. (B) Western blot analysis of ERK phosphorylation in control hADSC or hADSC treated with 10 μM U0126 for 0.5 h. (C) Western blot analysis of ERK phosphorylation in hADSC treated with 5 μM quercetin in time ranged from 0.5 to 24 h. Western blot signal was analyzed by an image analysis program (Multi Gauge V3.0, FUJIFILM). Data were presented as the relative ratio of phosphorylated ERK (p-ERK) to total ERK (t-ERK) signal of the corresponding samples (mean ± S.E.M., n = 3). $^*P < 0.05$ compared with control hADSC (cont.).

3.2. *Changes of gene expression in quercetin-treated hADSC*

In order to gain understanding into the action of quercetin on hADSC at the molecular level, we determined the effects of quercetin on the expression of the genes involved in osteogenesis, via RT-PCR. Three days of quercetin pretreatment in control culture media induced an increase in BMP2, Runx2, and osteopontin expression, which was confirmed by real time PCR analysis (Fig. 3A and B, Table 1). The quercetin-pretreated cells evidenced increases in alkaline phosphatase expression on the 3 days after exposure of the cells to osteogenic medium, whereas alkaline phosphatase expression increased on day 6 after the induction of differentiation of control hADSC. RT-PCR analysis also showed that BMP2 expression increased significantly during osteogenic differentiation in the quercetin-pretreated hADSC, compared with control hADSC (Fig. 3C and D).

3.3. *Quercetin action on proliferation and survival of hADSC*

MSC undergo mitotic clonal expansion upon exposure to differentiation media [4]. As mitotic clonal expansion can affect differentiation characteristics, we attempted to determine whether quercetin had any measurable effects on cell proliferation. Forty-eight hours of quercetin treatment inhibited the proliferation of hADSC in a dose-dependent manner, but did not affect on cell viability which was analyzed by trpan blue exclusion test (Fig. 4).

3.4. *Signaling mechanism of quercetin-induced osteogenic differentiation*

As the mitogen activated protein kinase (MAPK) pathway has been suggested to be involved in the osteogenic differentiation of MSC [23], we assessed the effects of U0126 on the quercetin-induced increase in osteogenic differentiation in the hADSC. U0126 has been known to be a specific inhibitor of MEK [24,25] and to be used for role of MAPK pathway on biological functions *in vitro* and *in vivo* [26–28]. Control hADSC or hADSC pretreated with 5 μM quercetin were induced into osteogenic differentiation in the absence or presence of 10 μM U0126 for 2 weeks. Quercetin-pretreated hADSC failed to show increased osteogenic differentiation in osteogenic media containing 10 μM U0126 (Fig. 5A). Western blot analysis showed that U0126 inhibited ERK phosphorylation in hADSC (Fig. 5B).

In order to assess the direct correlation of quercetin action with ERK activation, we determined the effects of quercetin on the ERK phosphorylation. hADSC (about 50% confluency) were incubated with 5 μM quercetin for 30 min to 24 h in control culture media. Fig. 5C showed that the 30 and 60 min treatment of quercetin significantly inhibited ERK phosphorylation ($p < 0.05$), which was gradually recovered to normal level upto 24 h. We also determined ERK phosphorylation during osteogenic differentiation. In this experiment, cells were cultured for 3 days in control or quercetin-containing media and reached at confluent state before induction of differentiation. Cell lysates were obtained from control hADSC or quercetin-pretreated hADSC before induction of osteogenic differentiation and from cells at 3, 6, and 12 days after induction of osteogenic differentiation. Although quercetin itself inhibited ERK phosphorylation transiently, ERK activation during osteogenic differentiation was significantly increased in quercetin-pretreated hADSC than control (Fig. 6).

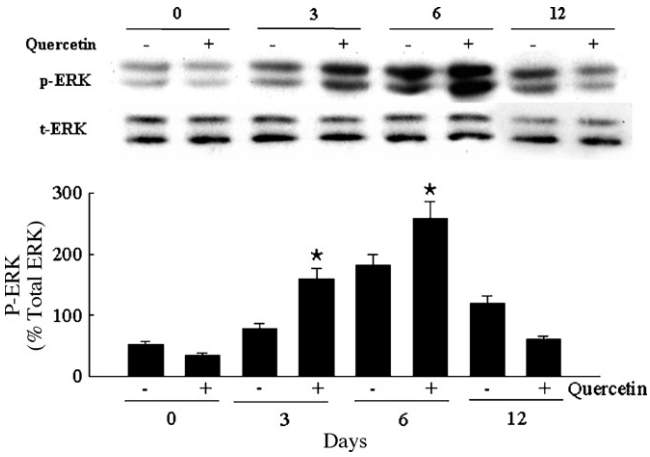

Fig. 6 – Effect of quercetin pretreatment on ERK acvitity during the osteogenic differentiation of hADSC. Quercetin-pretreated hADSC showed higher ERK phosphorylation during differentiation than naïve cells. hADSC were cultured for 3 days with control or 5 μM quercetin-containing media after plating. Cell number for initial plating was adjusted to reach confluent state after 3 days incubation. And then osteogenic differentiation was induced for 12 days. Lysates were prepared at the indicated times and were subjected to immunoblot analysis using phosphospecific and non-activated ERK antibodies. Data were presented as the relative ratio of p-ERK to t-ERK signal of the corresponding samples (mean ± S.E.M., $n = 3$). $^{*}P < 0.05$ compared with control hADSC at the indicated days.

Flavonols have previously been reported to exhibit estrogenic activity [29]. We determined the effects of an estrogen receptor antagonist on the quercetin and estrogen-induced increases in osteogenic differentiation. ICI182780 interacts with estrogen receptors specifically [30], and inhibit estrogen action *in vitro* and *in vivo* [31]. The addition of ICI182780 in differentiation media had no detectable effect on quercetin-induced osteogenic differentiation, although it inhibited 17β-estradiol-induced osteogenic differentiation (Fig. 7).

3.5. *Regeneration of skull-defects by hADSC transplantation*

In order to determine whether quercetin-pretreated hADSC evidenced superior bone regeneration abilities *in vivo*, we transplanted naive or quercetin-pretreated hADSC into 4 mm skull-defects in nude mice. The hADSC were pretreated with 5 μM quercetin for 3 days prior to transplantation. The identical number of naive or quercetin-pretreated cells (10^6 cells) were then added to 1 mm thickness collagen pads [32]. Five weeks after the transplantation, bone regeneration was found to have increased significantly in the quercetin-pretreated hADSC transplanted animals (Fig. 8).

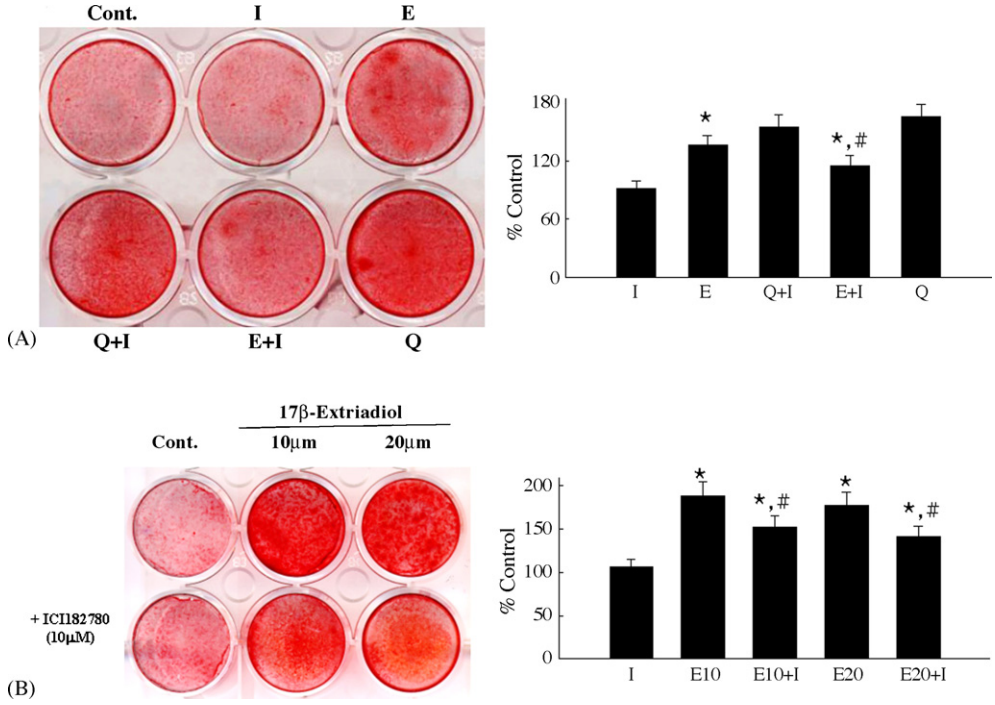

Fig. 7 – Effect of ICI182780 on 17-β estradiol- and quercetin-induced osteogenic differentiation of hADSC. Quercetin-induced osteogenic differentiation was not inhibited by an estrogen receptor antagonist. (A) hADSC were pretreated with 5 μM quercetin or 10 μM ICI 182780 or 10 μM 17-β estradiol for 3 days before induction of osteogenic differentiation. I: ICI 182780; E: 17-β estradiol, and Q: quercetin (B) cells were grown to confluence and then induced osteogenic differentiation by differentiation media containing 10 μM ICI 182780 or 17-β estradiol or both. Osteogenic differentiation was determined by alizarin red S staining of calcification deposits within the cell monolayer. The quantitation of osteogenic differentiation was performed by determination of density and area of alizarin red S staining with an image analysis program (Multi Gauge V3.0, FUJIFILM). Data were presented as a percentage of control (mean ± S.E.M., $n = 3$). $^{*}P < 0.05$ compared with control hADSC. $^{\#}P < 0.05$ compared with 17-β estradiol alone.

4. Discussion

Quercetin showed variable effects on osteogenic differentiation in various osteoblastic cells [16–18]. In this study we demonstrated that the flavonols effected an increase in the calcification of the extracellular matrix in hADSC, without any significant cytotoxic effect on the cells, and that the strongest stimulatory effect among the agents to be examined can be observed with quercetin treatment. The addition of reagents for modulation of stem cell functions can raise safety inssues in clinical trials of hADSC transplantation. The data in this study demonstrated that the pretreatment with 5 μM quercetin prior to the induction of differentiation had stimulatory effects on *in vitro* osteogenic differentiation in hADSC. Increased osteogenic differentiation potential of quercetin-pretreated cells was confirmed by increased expression of alkaline phosphatase and BMP2 genes during differentiation than naive cells, and by better bone regenerating ability in the skull defect model of nude mice than control cells.

Our RT-PCR and real time PCR data showed that quercetin pretreatment itself induced an increase in the expression of osteogenic differentiation-related genes, including osteopontin, Runx2 and BMP2 under control culture conditions. The roles of these genes on osteogenic differentiation have been fairly well-defined in osteoblasts [33] and MSC [34–37]. These results indicate that quercetin pretreatment in hADSC induces the expression of osteogenesis-related genes, which facilitates the response to osteogenic signals.

The data in this study showed that quercetin inhibited cell proliferation in a dose-dependent manner without affecting cell viability. Quercetin-induced inhibition of cell proliferation has been reported in various cancer cells [38,39]. Recent studies showed that the antiproliferative effect of quercetin is mediated via inhibition of PI3 kinase in cancer cells [40,41]. A recent microarray study of human MSC osteogenesis has demonstrated a downregulation of key proteins involved in proliferation upon osteogenic differentiation, such as the Wnt-responsive gene myc [42], and there is a naturally occurring transition from a more proliferative to a less proliferative phenotype during osteogenic differentiation [43]. The finding in this study that quercetin treatment in control culture media increased expression of osteogenesis-related genes indicates the lineage commitment of hADSC by the pretreatment of quercetin, which partly explains quercetin-induced decrease of hADSC proliferation. However, we cannot exclude the possibility that inhibition of proliferation itself may induce differentiation because of inverse relationship between proliferation and differentiation in stem cells. The relationship between differentiation and proliferation in osteogenesis is complex, because overexpression of osterix

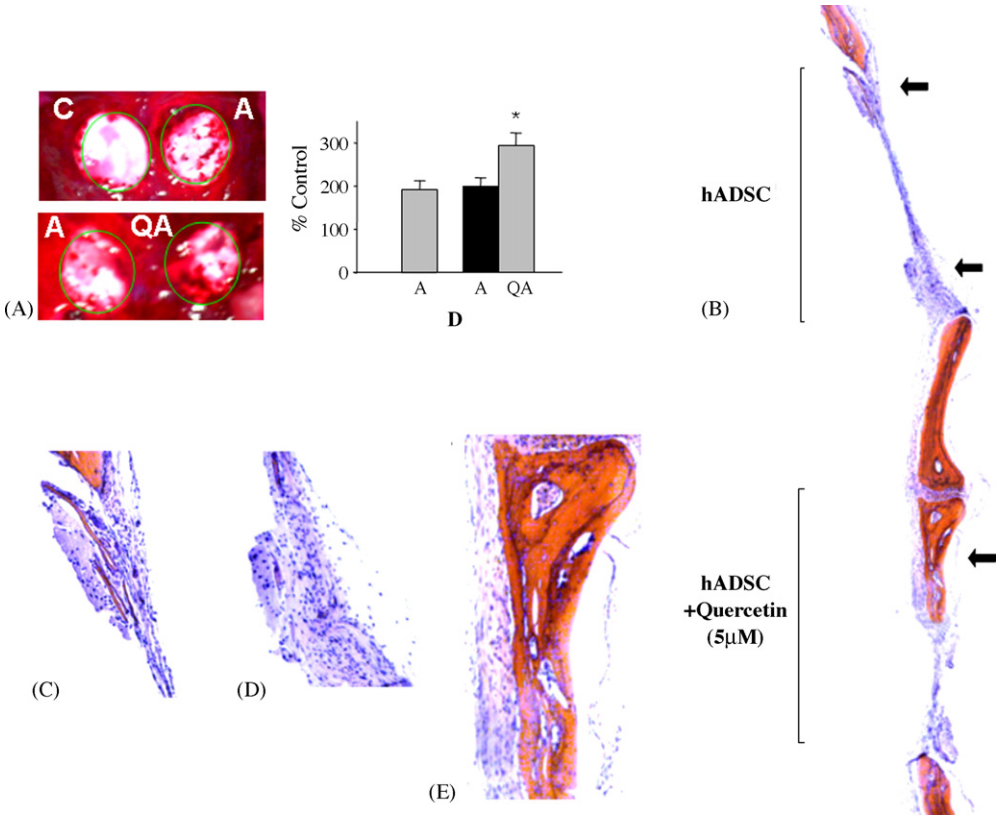

Fig. 8 – Effect of quercetin pretreatment on healing of calvarial defects in nude mouse by hADSC. Alizarin red and H&E staining identified increased bone formation in quercetin-pretreated hADSC transplanted side, as compared to control hADSC implanted side. Alizarin red (A) and histological analysis (B–E) of skull specimens at 5 weeks after transplantation of collagen scaffold alone (C), hADSC (A) or quercetin-treated hADSC (QA). (A) Skulls were stained with 1 mg/ml alizarin red for 12 h in order to visualize bone formation. A circle of standardized area was positioned into the defect and highlighted areas, in which the bone was mineralized; these areas were quantified as a function of the number of pixels within the area. The quantified data were presented as a percentage of collagen transplanted side (mean ± S.E.M., $n = 3$). $^{*}P < 0.05$ compared with hADSC transplanted side. Panel (B) shows the area of the defect taken with an original magnification of 50×. Panels (C), (D) and (E) show higher magnification views of the regions indicated by the black arrow in (B).

[44] or the treatment of biphosphonates [45] enhances both proliferation and osteogenic differentiation of MSC.

The interaction between flavonols and flavonoids with estrogen receptors has been fairly well-documented [46,47]. Kuiper et al. [46], who studied the estrogenic potency of several phytoestrogens, demonstrated that kaempferol, and to a lesser extent quercetin, have the capacity to bind to both ER subtypes. In this study, quercetin evidenced effects on osteogenic differentiation in hADSC superior to those of kaempferol, thereby suggesting that the action of quercetin is not related to the activation of ER. The independency of the quercetin effect with estrogenic potency was further supported by the finding that ICI182780 failed to inhibit quercetin-induced osteogenic differentiation, but did exert an inhibitory effect on estradiol-induced osteogenic differentiation.

In this study, the addition of an MEK inhibitor U0126 inhibited osteogenic differentiation in naive cells as well as in quercetin-pretreated cells and induction of osteogenic differentiation increased ERK phosphorylation, suggesting that the ERK pathway is involved in the osteogenic differentiation of hADSC. Roles of ERK activation on osteogenic differentiation in bone marrow-derived MSC has been well documented [48,49]. Several reports have indicated that estradiol activates the MAPK pathway via nongenomic action, through the rapid interaction of the estradiol-ER complex [50], and that flavonols can activate the MAPK pathway via interaction with the ER [18]. However, the results of our experiments indicated that quercetin itself failed to activate ERK phosphorylation in hADSC at control culture condition (Fig. 5C), although quercetin-pretreated hADSC showed increased ERK phosphorylation at days 3 and 6 after induction of osteogenic differentiation. Increased ERK phosphorylation at days 3 and 6 after exposure of osteogenic medium can be explained by quercetin-induced lineage commitment of quercetin-pretreated hADSC via ERK-independent mechanisms. Lineage-committed hADSC, in turn, results in increased ERK activation during the induction of differentiation. Quercetin-induced lineage commitment of hADSC in control culture media is supported by the findings that quercetin pretreatment increased expression of osteogenesis-related genes in control culture media, increased osteogenic differentiation during the induction of differentiation in the absence of quercetin, and

the transplantation of quercetin-pretreated cells without induction of differentiation showed better *in vivo* bone regeneration than transplantation of naïve hADSC.

The autologous nature of mesenchymal stem cells, together with their putative multipotentiality, may render these cells an excellent choice for many future tissue engineering strategies and cell-based therapies. MSC have been shown to differentiate into both osteogenic and chondrogenic tissues *in vivo* [51–53], and preliminary data indicate that these cells can be used to repair both bony and cartilaginous defects [54,55]. However, due to the fact that the *in vivo* bone regeneration ability conferred by these techniques remains insufficient, the development of techniques for the enhancement of *in vivo* bone regeneration with MSC via either genetic modification or BMP-2 treatment continues. Quercetin is one of the most abundant flavonoids in the human diet. Measured concentrations of quercetin glycoside in human plasma tend to range between 5×10^{-7} and 1.6×10^{-6} M [56]. Therefore, quercetin is considered as a safe natural product for tissue engineering.

In conclusion, the pretreatment of quercetin, a natural product, in hADSC enhances their *in vitro* osteogenic differentiation through ER-independent mechanisms, and the transplantation of quercetin-pretreated cells induces bone regeneration in a skull defect model of nude mice efficiently. These results indicate that quercetin-pretreatment protocol can be used in further clinical trials of *in vivo* bone engineering using MSC.

Acknowledgments

This work was supported by a grant (R01200600010525020006) from the ministry of science and technology and the MRC program of MOST/KOSEF (R13-2005-009).

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