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Interaction of Mesenchymal Stem Cells and Osteoblasts for *in vitro* Osteogenesis

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It has recently been reported that bone marrow-derived mesenchymal stem cells (MSCs), which are systemically administrated to different species, undergo site-specific differentiation. This suggests that the tissue specific cells may cause or promote the differentiation of the MSCs toward their cell type via a cell-to-cell interaction that is mediated not only by hormones and cytokines, but also by direct cell-to-cell contact. In this study, in order to assess the possible synergistic interactions for osteogenesis between the two types of cells, the MSCs derived from rabbit bone marrow were co-cultured with rat calvarial osteoblasts in direct cell-to-cell contact in a control medium (CM) and in an osteogenic medium (OM). The cell number, alkaline phosphatase activity, and amount of calcium deposition were assayed in the cultures of MSCs, osteoblasts, and co-cultures of them in either OM or CM for up to 40 days. The cell numbers and the alkaline phosphatase activities in the co-culture were somewhere in between those of the osteoblast cultures and the MSC cultures. The amounts of deposited calcium were lower in the co-culture compared to those of the other cultures. This suggests that there are little synergistic interactions during osteogenesis *in vitro* between the rat osteoblasts and rabbit MSCs.

Key Words: Mesenchymal stem cells, osteoblasts, co-culture, osteogenesis, mineralization

INTRODUCTION

Friedenstein initially reported that clonogenic stromal cells could be derived from the bone marrow.[1] The most striking feature of the cells was that they had the ability to differentiate into small colonies resembling bone or cartilage. Since then, bone marrow derived mesenchymal stem cells (MSCs) have been actively studied and found to have the tripotential of differentiating into osteoblasts, chondrocytes, and adipocytes.[2] In addition, they can differentiate into myoblasts,[3,4] cardiomyocytes[5-7] and neurons.[8] MSCs are relatively easy to isolate from the small aspirates of bone marrow, which can be obtained under local anesthesia. They are also relatively easy to expand in cultures and transfect with exogeneous genes.[9] By virtue of these merits, MSCs have become promising vehicles for gene therapy,[9] cell therapy [9,10] and tissue engineering, particularly for bone tissue engineering.[11-13] MSCs have been isolated and characterized for their tripotentiality to undergo osteogenic, chondrogenic and adipogenic differentiation in humans, mice and rats.[2,14-17] In rabbits, MSCs have been poorly characterized by their potential to differentiate into the above three lineages particularly regarding adipogenic differentiation.

Several experiments on the behaviors of MSCs after a systemic injection therapy have been carried out. When the MSCs from a line of transgenic mice expressing a mutated type I collagen gene were systemically infused into the marrow ablated, isogenic mice, either donor MSCs or their progeny were detected in the liver, thymus, lung, marrow, and spleen.[9] Moreover, the expression of the mutated marker gene was detected in the cultured bone cells but not in the cartilage cells. The results suggested that the progeny of MSCs expressed genes in a tissue specific manner because the marker gene was for type I collagen, a gene that is expressed in bone but not in cartilage. When the human MSCs were

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systemically administered into fetal lambs *in utero*, they underwent site-specific differentiation into chondrocytes, adipocytes, myocytes and cardiomyocytes, as well as into bone marrow stromal cells and thymic stroma.[18] In addition, a co-culture of differentiating embryonic mouse stem cells with fetal mouse osteoblasts without direct cell-to-cell contact promoted the formation of mineralized bone nodules than those cultured without the fetal osteoblasts.[19]

Considering the above reports, it is possible that osteoblasts can induce or potentiate the osteogenic differentiation of MSCs by a cell-to-cell interaction. However, the osteogenesis of mesenchymal stem cells was not induced by a co-culture with osteoblasts in a membrane separated *trans*-well culture chamber without direct cell-to-cell contact.[20] As in the above experiments, the *in vitro* studies of the interaction between osteoblasts and MSCs were carried out without direct cell-to-cell contact. However, the cell-to-cell interaction is mediated not only by hormones or cytokines, but also by this direct cell-to-cell contact.[21] In addition, the cell-cell interactions via the cell adhesion molecules, particularly cadherins, are critically involved in the different aspects of bone remodeling, including osteoblast differentiation.[22]

In order to clarify the effect of a co-culture of mesenchymal stem cells and osteoblasts on the *in vitro* osteogenesis under direct cell to cell contact, the MSCs derived from rabbit bone marrow were co-cultured with rat calvarial osteoblasts in direct cell-to-cell contact in a control medium and in an osteogenic medium to assess if there is a synergistic interaction for osteogenesis between the two cell types. Before the co-culture experiment, the isolated cells from the rabbit bone marrow were characterized by their potential to differentiate into osteoblasts, chondrocytes and adipocytes. Cellular proliferation, alkaline phosphatase, and calcium deposition were measured to compare the osteogenic function.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma (Saint Louis, Mo, USA) unless otherwise stated. The kits for assaying the alkaline phosphatase activity and calcium concentration were purchased from Sigma Diagnostics (Saint Louis, Mo, USA). The 0.25% trypsin in 0.02% sodium ethylenediaminetetraacetic acid (EDTA) was acquired from JRH Bioscience (Lenexa, KS, USA). The Dulbecco's phosphate buffered saline (DPBS) and fetal bovine serum (FBS) were obtained from Life Technologies (Grand island, NY, USA). The culture flasks,[24] well multi-dishes and 15-ml polypropylene conical centrifuge tubes were purchased from Nalge Nunc International (Roskilde, Denmark). The rotary incubators were bought from Heidolph (Germany). The optical density was measured using a microplate spectrophotometer (Molecular devices, Sunnyvale, CA, USA). All optical densities were measured from triplicate aliquots and their means were used for analysis.

Isolation and culture of MSCs and osteoblasts

All animal experiment procedures were managed in accordance with the Guidelines and Regulations for Use and Care of Animals in Yonsei University. The rabbit MSCs were obtained from adult female white New Zealand rabbits aged between 8 months and 1 year, weighing between 2.5 kg and 3.3 kg, using a modification of the method previously reported.[23-25] The rabbits were anesthetized with an intramuscular administration of ketamine (50 mg/kg) and xylazine (10 mg/kg). Under general anesthesia, the bone marrow was aspirated from the tibia using a 10ml syringe containing 0.1 mL heparin (3000 U/mL saline solution), with a 16-gauge needle. The marrow aspirates were suspended in DPBS, centrifuged, and resuspended in a control medium (CM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotics/antimycotics (final concentration: penicillin 100 units/mL, 100μg/mL streptomycin and 0.25 μg/mL amphotericin B) and 10% FBS. The cells were plated at a concentration of 2×10^6 nucleated cells/cm^2. The non-adherent cells were removed by changing the medium. The colonies grew quickly between days 5 and 9, and the cells were passaged when the cell density approached 80 - 90% confluence. The second-passage cells

were used for the rest of the experiments.

The rat calvarial osteoblasts were obtained from 4-day-old Sprague-Dawley rat pups using the methods previously described.[26,27] The fourth-passage cells were used for the rest of the experiments.

Confirmation of the potential of the isolated rabbit marrow stromal cells to differentiate into chondrocytes and adipocytes

Adipogenic and chondrogenic differentiation was additionally induced and evaluated to confirm that the isolated cells from the rabbit bone marrow were mesenchymal stem cells, which have at least the tripotential of being able to differentiate into adipocytes, chondrocytes, and osteoblasts. Adipogenic differentiation was induced through a culture in adipogenic media as previously described.[2] Differentiation was demonstrated by the intra-cytoplasmically accumulated lipid vacuoles, which were stained by Oil Red O. Chondrogenic differentiation was induced by culturing in chondrogenic media, as previously described[28,29] and confirmed by an Alcian blue stain.

Comparative osteogenic function assay of the cultures of osteoblasts, MSCs and co-culture of them

The MSCs and osteoblasts were trypsinized and replated at a concentration of 5.7×10^3 cells/well (i.e. 3.0×10^3 cells/cm^2) in 24 well multi-dishes. In the case of an osteoblast and MSC co-culture, each type of cell was seeded at a half density, i.e. 2.85×10^3 cells/well each, in order for the sum of both cell concentrations to be same as that of the other wells. The co-cultures were carried out after a complete mixing of the two kinds of trypsinized cells by stirring. Osteoblasts, MSCs and co-cultures were cultured in an osteogenic medium (OM) and control medium (CM). The OM consisted of the CM supplemented with 0.1 μM dexamethasone, 50 μM ascorbic acid 2-phosphate, 10 mM β-glycerophosphate.[30] Their osteogenic functions were quantified by an alkaline phosphatase activity assay and a calcium deposition assay, and qualitatively measured by Alizarin Red S stain.

Proliferation assay

The cell numbers were measured in triplicate cultures using a modification[30,31] of the crystal violet dye-binding method.[32] The cultures were rinsed with Tyrode's balanced salt solution and fixed in 1% glutaraldehyde (v/v) in Tyrode's balanced salt solution for 30 min, rinsed twice with deionized water (DW), and then air-dried. The cultures were then stained with 0.2% crystal violet (w/v) in 2% ethanol for 15 min. After the unstained dye was carefully rinsed three times with DW, the crystal violet dye was extracted from the cells by a 4hr rotary incubation at room temperature with 1% sodium dodecyl sulfate (SDS) in 50% ethanol. The extracts were properly diluted and the optical density was measured at 595 nm. The absorbances were converted into absolute cell numbers based on an established standard curve.

Alkaline phosphatase activity

The alkaline phosphatase (ALP) activities were measured using a modification of a previously reported method.[24] Briefly, the cultures were rinsed twice with Tyrode's salt solution (Sigma, Saint Louis, Mo, USA). The cell layers were covered by 0.7 ml of 5 mM p-nitrophenyl phosphate in an alkaline buffer solution at 37℃ for 15 min on a rotary incubator. 100 μl of the reaction solution were transferred to 96 well plates, which previously contained 100 μl of 1 N NaOH solution in each well. The alkaline phosphatase enzyme activity was calculated by measuring the absorbance of the p-nitrophenol product at 405 nm. A standard curve was obtained from successive dilutions of a p-nitrophenol standard solution. Each alkaline phosphatase activity was normalized by the cell number that was determined by the crystal violet method, as previously described.[30]

Calcium assay

The deposited calcium was measured using a previously reported method.[30] The cultures were rinsed with Tyrode's salt solution and fixed with 1% (v/v) glutaraldehyde in Tyrode's for 30 min. Following fixation, the cultures were rinsed twice

with distilled water, and the calcium was extracted with 1ml of 0.6 N HCl per well. The well plates containing this solution were sealed with parafilm in order to prevent the solution from drying. They were placed on a rotary shaker and maintained at a speed of 50 rpm overnight. Aliquots of the extract were properly diluted and added to the wells of a 96-well culture dish (3 wells/sample). Reagents from a commercial calcium assay kit (Sigma Kit #587) were then added to the wells, and the absorbance was read at 575 nm using a microplate reader. The calcium concentration was calculated from a standard curve generated from a series dilution of a calcium standard solution. Each level of calcium deposition was normalized by the cell number that were measured using the crystal violet method, as previously described.[30]

Alizarin red S staining

The cultures were stained using a previously described method with Alrizarin Red S.[33] Briefly, the cultures were rinsed with Tyrode's salt solution, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), and rinsed with distilled water. The cultures were then stained with 40 mM Alizarin red S for 10 minutes at room temperature and washed with distilled water and PBS (pH 7.2).

Statistical analysis

All experiments were carried out in triplicate. The results from various experiments are reported as a mean ± standard deviation. The significance levels were determined by ANOVA or a Student's t-test. Multiple comparisons were carried out using the Tukey method. All statistical calculations were done using the SAS system for Windows (version 8.00, SAS Institute Inc.). A p-value < 0.05 was considered significant.

RESULTS

MSCs from rabbit bone marrow

The initial adherent cells isolated from the rabbit bone marrow grew as spindle- or stellate-shaped cells, which developed into visible colonies 3 to 5 days after the initial plating. They remained dormant for 2 to 4 days, then began to multiply rapidly and reached confluence on the 8th to 10 th day. The floating cells were removed from the medium changes and subsequent passaging. After the second passage, the adherent cells appeared to have a more uniform fibroblast-like appearance.

The cultured marrow-derived adherent cells underwent differentiation into adipocytes or chondrocytes depending on the culture condition. The MSCs cultured in the adipogenic media were found to have intra-cytoplasmic lipid vacuoles stained by Oil Red O (Fig. 1). The MSCs cultured in chondrogenic media formed cellular pellets stained by Alcian blue (Fig. 1).

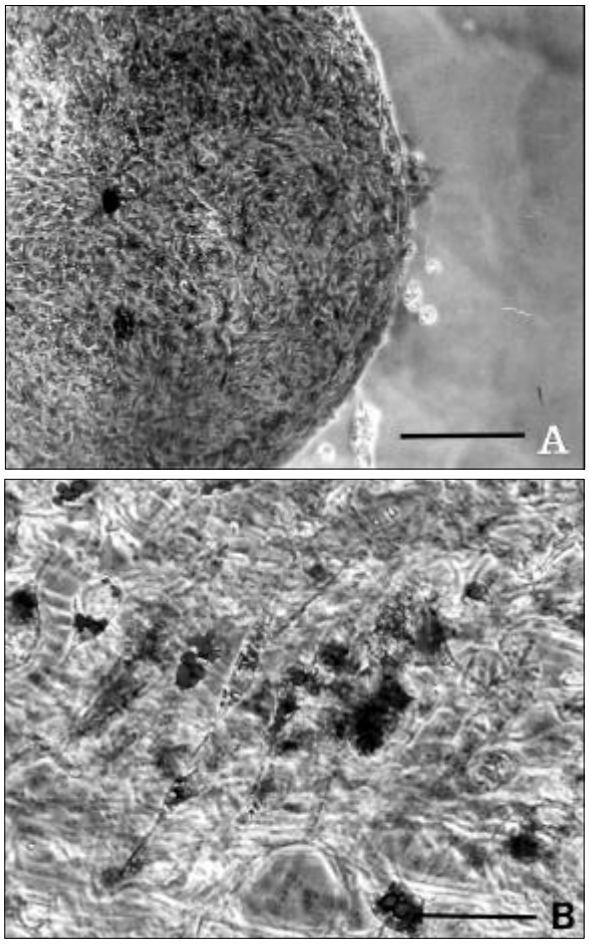

Fig. 1. Rabbit bone marrow derived mesenchymal stem cells underwent differentiation into chondrocytes, as stained by Alcian blue (A) and adipocytes, as stained by Oil Red O (B). The scale bar indicates 150 μm (A) and 75 μm (B).

Comparative osteogenic function assay

Proliferation

Two-way ANOVA analysis showed that there were significant interactions for the cell number between the culture media and cultured cell types. Accordingly, one-way ANOVA was used to analyze the effect of the cell type and the culture medium on cellular proliferation. The resulting cell numbers of the osteoblasts and MSC co-culture ranged from those of the MSCs and those of the osteoblasts either in the OM or CM on all assayed culture days (Fig. 2). The cell numbers of the co-cultures in the CM did not appear to be somewhere between the cell numbers from the osteoblasts cultures and the MSCs cultures on the 8th day. However, these differences were not statistically significant ($p > 0.4$). The numbers of MSCs in the OM were greater than those of the MSCs in CM on all days ($p < 0.05$) except day 1. The MSCs in the OM tended to detach spontaneously from the culture dish after day 30 (Fig. 5). This detachment might be due to the formation of membranes, which were expected to be composed of cells and extracellular matrices, mainly collagen. The cells from the other cultures were not detached from the culture dish.

Alkaline phosphatase activity

All ALP activities rose until the 5th culture day ($p < 0.04$), reached a peak between day 5 and 8, and decreased thereafter (Fig. 3). The ALP acti-

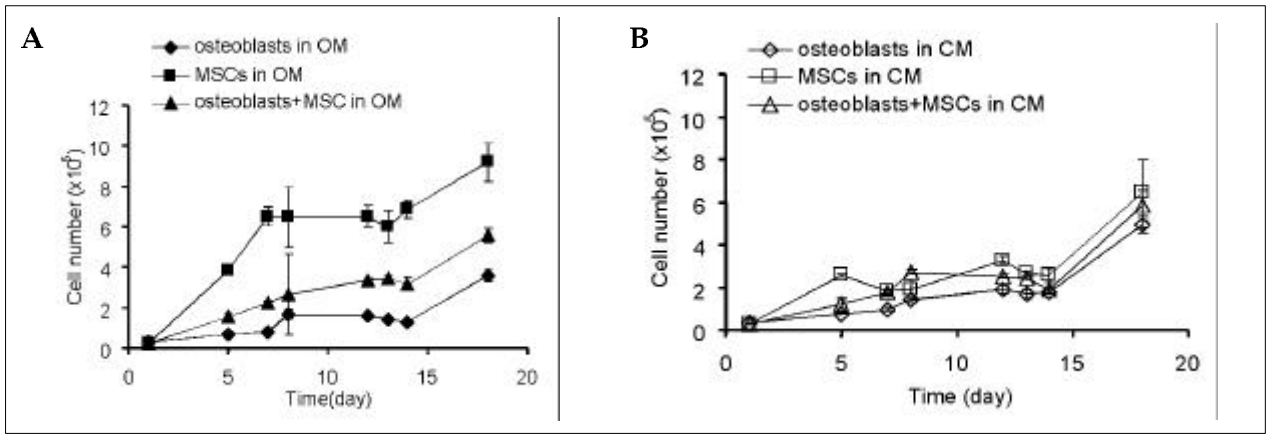

Fig. 2. Cell proliferation in rat osteoblasts cultures, the rabbit MSCs cultures, and co-culture of the two kinds of the cells. The numbers of osteoblasts, MSCs, and the co-cultures in the osteogenic medium (OM) (A) and those in the control medium (CM) (B) were shown. The results represent the mean ± standard deviation of triplicate cultures.

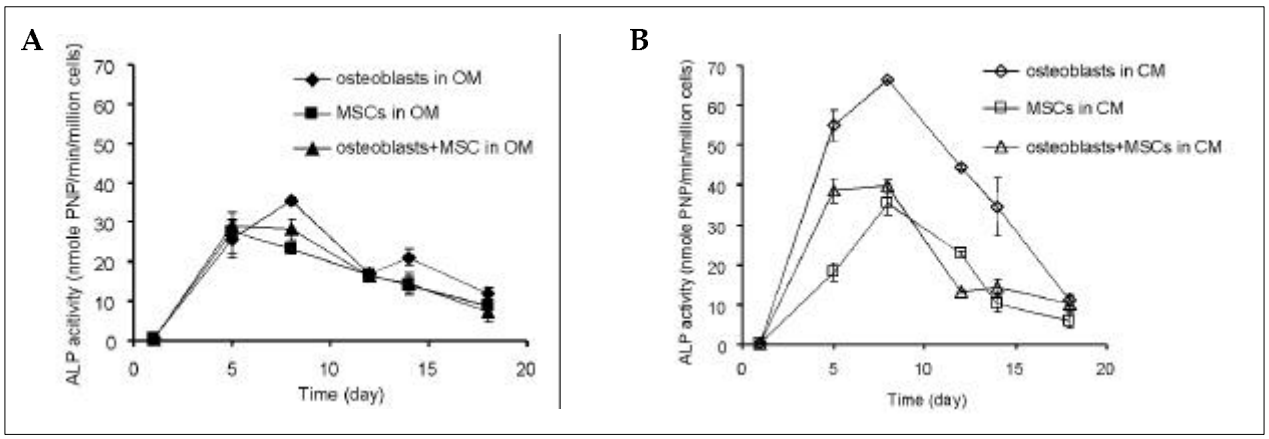

Fig. 3. Alkaline phosphatase (ALP) activities in rat osteoblasts cultures, rabbit MSCs cultures, and co-cultures of the two types of cells in the osteogenic medium (OM) (A) and in the control medium (CM) (B). The results represent the mean ± standard deviation of triplicate cultures.

vities from day 5 or 8 of each culture group were significantly higher than the other days ($p<0.05$). There was no significant difference between the ALP activities from day 5 and day 8 in all culture groups ($p>0.1$) except for the cultures in the CM. When the activities of day 5 were compared with that of day 8, each culture groups showed $p>0.1$ with the exception of MSC cultures in the CM. The ALP activity in the MSC culture in the CM on day 8 was significantly greater than those on day 5. The ALP activities decreased after the 8th day. It should be noted that either in the OM or in the CM, the ALP activities of the co-cultures were somewhere between those of the osteoblast cultures and those of the MSCs cultures on all days assayed ($p>0.1$) except day 12 in the CM ($p<0.05$).

Mineralization

Significant calcium deposition was not observed in any culture in the CM until day 40. Significant calcium deposition began to show initially in the MSC culture in the OM (13th day, $p<0.05$) followed by the osteoblast culture in the OM (23rd day, $p<0.02$) and the co-cultures in the OM (40th day) ($p<0.01$) (Fig. 4). The amount of calcium deposition greatly increased between day 29 and 40 in all cultures in the OM. Calcium deposition started as a nodular pattern in all the cultures in the OM. Calcium was almost uniformly distributed in the osteoblasts in the OM on day 40 whereas a noncalcified area was observed in both the MSCs and the co-culture in the OM (Fig. 5 and 6). In certain areas of the MSC cultures in the OM, calcium deposition stained by Alizarin Red S was not observed until day 40. In some of these non-mineralized culture areas, cells containing intracytoplasmic lipid vacuoles were observed, which suggests adipogenic differentiation.

DISCUSSION

Isolated cells from rabbit bone marrow showed the characteristics of MSCs

Initially, the isolated adherent cells were observed as heterogeneous groups of cells. However, they became homogeneous as the cells continued to proliferate, and were more homogeneous after subsequent passaging. The initial heterogeneous morphology may be due to the presence of other types of adherent cells such as macrophages, lymphocytes, and endothelial cells.[34] Depending on the culture media and conditions, the cells adhering to the culture flasks, which were isolated from the rabbit bone marrow, underwent osteogenic, chondrogenic, and adipogenic differentiation. In other words, the isolated cells exhibited mesenchymal stem cell characteristics, which was similar to the results obtained from human MSCs.[2]

Interactions between MSCs and osteoblasts for *in vitro* osteogenesis

The cell numbers and ALP activities of the co-culture were somewhere between the range of those of the osteoblasts and MSCs cultures on most assayed days. This suggests that there are no

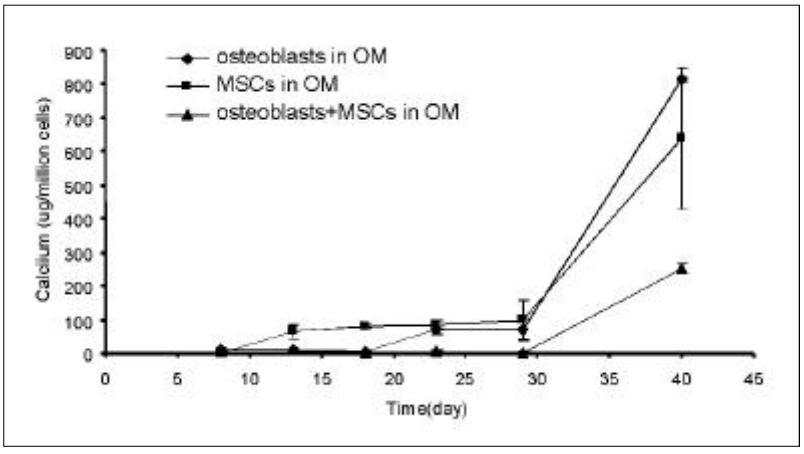

Fig. 4. Calcium deposition in rat osteoblasts cultures, rabbit MSCs cultures, and co-cultures of the two kinds of the cells in the osteogenic medium (OM). The results represent the mean ± standard deviation of triplicate cultures.

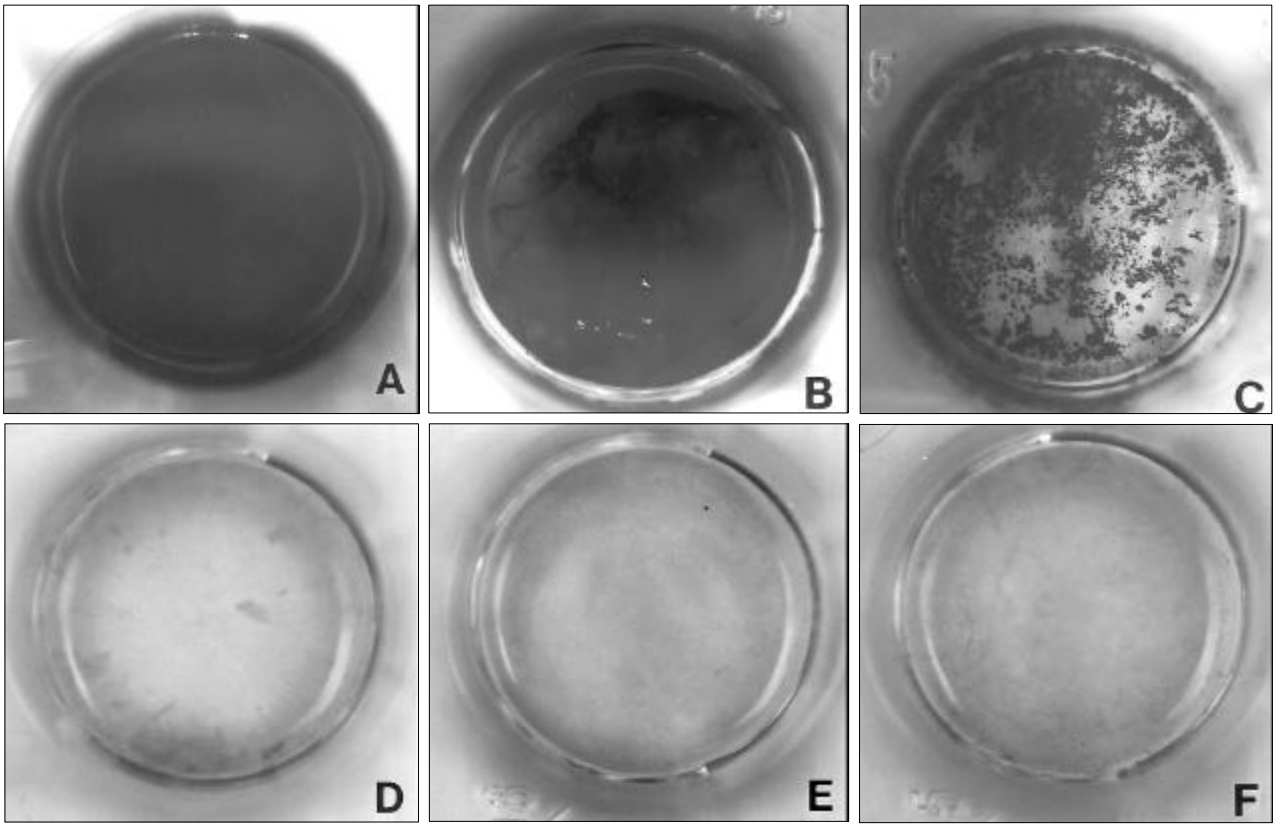

Fig. 5. Alizarin red S staining of rat osteoblasts cultures (A, D), rabbit MSCs cultures (B, E), and co-cultures (C, F) of the two kinds of the cells grown in the osteogenic medium (OM) (A-C) and in control medium (CM) (D-F) in 24 well multi-dishes on the 40th day of culture. Calcium was stained red by Alizarin red S.

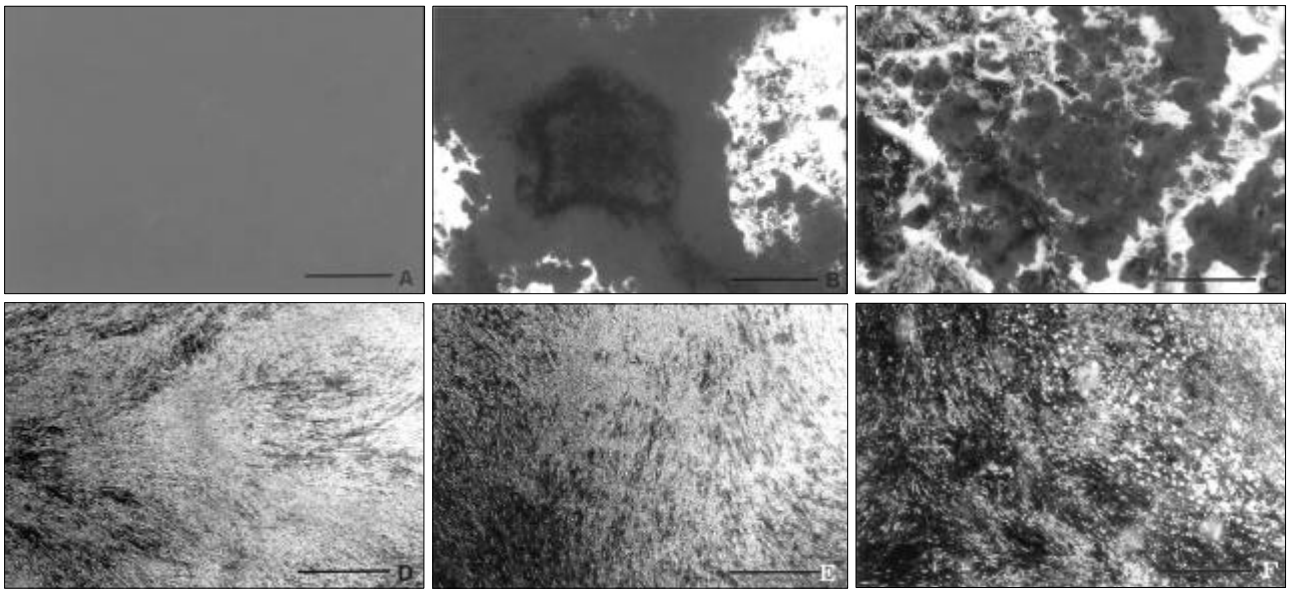

Fig. 6. Alizarin red S staining of rat osteoblasts cultures (A, D), rabbit MSCs cultures (B, E), and co-cultures (C, F) of the two types of the cells grown in the osteogenic medium (OM) (A-C) and in the control medium (CM) (D-F) in 24 well multi-dishes on the 40th day of culture. Calcium was stained red by Alizarin red S. Each scale bar indicates 500 μm.

significant interactions between the two cell types for osteogenesis *in vitro*. However, mineralization was detected later in the co-cultures in the OM than in either the osteoblasts or MSCs cultures, and the deposited calcium content was also lower in the co-cultures in the OM than in the cultures of either osteoblasts or MSCs. This indicates that there might be no significant synergistic interaction between these two cell types for *in vitro* osteogenesis.

As previously mentioned, Gerstenfeld LC, et al recently reported that osteoblasts do not induce the osteogenic differentiation of MSCs by humoral factors such as hormones or cytokines.[20] They co-cultured the C3H10T½ mesenchymal stem cells with calvarial osteoblasts and chondrocytes derived from embryonic chickens in trans-well culture chambers separated by a membrane. In their experiments, osteogenesis of C3H10T½, as determined by the expression of the Cbfa1 and osteocalcin messenger RNA, was induced strongly in the C3H10T½ cells co-cultured with chondrocytes but it was not induced by a co-culture with osteoblasts. In addition, the ALP activity was not induced in the C3H10T½ cells co-cultured with osteoblasts but was induced the C3H10T½ cells co-cultured with chondrocytes. Our results were consistent or at least compatible with their results.

Friedenstein et al. proposed that there might be two diverse categories of osteogenic precursor cells such as the determined osteoprogenitor cells (preosteoblasts) and the inducible osteoprogenitor cells (multipotent stem cells).[35] They suggested that the decalcified bone and the humoral substances produced by the transitional epithelium might be the osteogenic inducer of the multipotent stem cells. Dexamethasone, ascorbate-2-phosphate and the bone morphogenetic proteins along with other growth factors have been proposed as osteogenic inducers of multipotent mesenchymal stem cells.[30,36-38] Considering our results, the osteoblasts might not be the major osteogenic inducer.

Difference in mineralization pattern between rat osteoblasts and rabbit MSCs

Even though mineralization was initiated earlier in the MSC cultures, the total amount of calcium deposition in the MSC culture for 40 days was similar to that of the osteoblasts cultures. In other words, the calcium content increased slowly in the MSC culture in the OM and increased sharply in the osteoblasts culture in the OM. The slowing of mineralization in the MSC culture may result from the spontaneous adipogenic differentiation of some of the MSCs and/or the spontaneous detachment of the osteogenically differentiated MSCs from the culture plate in the OM. As mentioned in the results section, some cells in the non-mineralized culture area appeared to undergo adipogenic differentiation even in the OM. These culture areas of the MSCs, which might have undergone differentiation toward adipocytes, led to the reduction of the overall MSC culture area undergoing differentiation toward osteoblasts, in which calcium phosphate can precipitate. In other words, the effective area where calcium can be deposited was larger in the osteoblast cultures than in the MSCs cultures. In addition, MSCs in the OM tended to detach spontaneously or at least effortlessly. However, the MSCs in the adipogenic media were not detached spontaneously from the culture plates. This suggests that osteogenically differentiated MSCs can detach easily from the culture plates. This detachment might be due to the formation of membranes that are composed of cells and extracellular matrices, mainly collagen. If this is true, this detachment leads to the relative increase in the number of adiogenically differentiated MSCs and the reduction or slowing of the total amount of calcium deposition.

Mineralization and cell density

Significant mineralization was initiated earlier in the MSC cultures in the OM than in the osteoblast cultures in the OM. This result may be attributed to the difference in the species (rat and rabbit) or to the difference in cell density. In this experiment, significant mineralization was observed around days 5 to 8 after the cell counts reached more than 2.9×10^6 cells/well (1.5×10^6 cells/cm^2) in either of the osteoblast or MSC cultures in the OM. Cultures of MSCs in the OM reached this critical cell density earlier than those of osteoblasts. The cell density plays a role in the process of bone formation.[39] Cellular condensation, a process of cell aggregation mediated by

mesenchymal-epithelial cell interactions, plays a crucial role during skeletogenesis, affecting both chondrogenesis and osteogenesis.[40] The three-dimensional growth of human osteoblasts in tissue-like aggregates is a prerequisite for *ex vivo* bone formation.[41]

Osteogenic differentiation and osteogenic medium effect

In this study, the number of MSCs in the OM was significantly greater than that of the MSCs in the CM during most of the culture days. This result is consistent with the results reported for human MSCs.[30,42] The character of stem cells can explain this. When a stem cell undergoes a commitment to differentiate, it often enters transient state of rapid proliferation.[43] Owing to the osteogenic supplements, the MSCs in the OM underwent a commitment to osteogenic differentiation and entered the rapid proliferation stage.

The ALP activities of the MSCs in the OM were similar to those of the MSCs in the CM, and this result is consistent with previous results reported for rabbit marrow-derived MSCs.[24] Solchaga observed that only 2 (8%) of the 25 MSC preparations from rabbits were sensitive to osteogenic supplementation and showed an increased ALP value compared to that of the MSC cultures in the CM. However, this result is different from the results on the marrow-derived MSCs from humans,[30,42,44,45] rats,[6,46] and dogs,[47] where the osteogenic medium caused an increase in ALP activity compared to the control medium.

Calcium deposition was observed in all cultures in the OM, but significant calcium deposition was not observed in any of the cultures in the CM. This result from rabbit bone marrow MSCs is consistent with those reported for humans,[42,44,45] rats[16] and dogs.[47] Although no von Kossa positive mineral deposition was previously described[24] in rabbit MSC cultures in the OM, our Alizarin Red S stain, von Kossa stain (data not shown), and calcium measurement demonstrates that significant calcification occurred in the rabbit MSC cultures in the OM.

In conclusion, MSCs were isolated from rabbit bone marrow, and the isolated cells exhibited the character of mesenchymal stem cells undergoing adipogenic, chondrogenic and osteogenic differentiation depending on the culture conditions. The MSCs were co-cultured with osteoblasts under a direct cell-to-cell contact, and cell number, ALP activity and mineralization was assayed and compared with those of individual osteoblast cultures and MSC cultures. The cell numbers and ALP activities in the co-cultures were somewhere between those of the MSC cultures and the osteoblast cultures. The level of mineralization of the co-culture was lower than the MSC culture and osteoblast culture. This suggests that under direct cell-to-cell contact between the two kinds of cells, there are little synergistic interactions in the *in vitro* osteogenesis between the rabbit MSCs and the rat osteoblasts.

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