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Original article

Rapamycin as an inhibitor of osteogenic differentiation in bone marrow-derived mesenchymal stem cells

Shinji Isomoto[1], Koji Hattori[1], Hajime Ohgushi[2], Hiroshi Nakajima[3], Yasuhito Tanaka[1], and Yoshinori Takakura[1]

[1] Department of Orthopaedic Surgery, Nara Medical University, 840 Shijyo-cho, Kashihara, Nara 634-8522, Japan
[2] Research Institute for Cell Engineering, National Institute of Advanced Industrial and Technology, Amagasaki, Japan
[3] Department of Orthopedic Surgery, Otemae Hospital, Osaka, Japan

Abstract

Background. An autograft of cultured bone marrow-derived mesenchymal stem cells has already been used in clinical practice. In those patients whose bone marrow cannot be used, a cell allograft with the use of immunosuppressant drugs will be an option in the future. However, little is known about the effects of immunosuppressant drugs on mesenchymal stem cells. This study assessed the effects of immunosuppressant drugs on osteogenic differentiation of mesenchymal stem cells and analyzed the manner in which immunosuppressant drugs modulate the osteogenic effect of dexamethasone.

Methods. Rat bone marrow cells were cultured with or without dexamethasone as an osteogenic supplement. In each experimental group, one of three immunosuppressants (rapamycin, cyclosporine A, or FK506) was added. As a control, cells were cultured without immunosuppressants. Histologically, mineralization was assessed by alizarin red S staining and phase-contrast microscopy. Biochemically, alkaline phosphatase activity, calcium content, and osteocalcin content were assessed.

Results. On histological analysis, no mineralized nodules were seen on alizarin red S staining or phase-contrast microscopy in the groups not treated with dexamethasone, except in the group that was treated with FK506. Mineralized nodules were seen in the groups treated with dexamethasone, except in the group that was treated with rapamycin. On biochemical analysis, it was found that, compared to the control group, rapamycin reduced alkaline phosphatase activity and the calcium content of mesenchymal stem cells; FK506 increased alkaline phosphatase activity, calcium content, and osteocalcin content; and cyclosporine A had negligible effects. Dexamethasone increased alkaline phosphatase activity, calcium content, and osteocalcin content, but these effects were decreased by rapamycin.

Conclusions. Rapamycin did not have an osteogenic effect on mesenchymal stem cells, but inhibited the effect of osteogenic differentiation induced by dexamethasone. In contrast, FK506 had an osteogenic effect on mesenchymal stem cells. Therefore, FK506 might be more useful than rapamycin in allogeneic transplantation of mesenchymal stem cells.

Offprint requests to: K. Hattori
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Introduction

Advances in tissue engineering have enabled the development of new procedures for bone regeneration using bone marrow-derived mesenchymal stem cells. It is widely known that, under the proper conditions, mesenchymal stem cells (MSCs) residing in bone marrow can differentiate into the osteogenic lineage. Therefore, MSCs combined with hydroxyapatite ceramics can be used clinically to fill bone defects. We have also initiated the clinical use of tissue-engineered artificial joint replacements using alumina ceramics covered with cultured MSCs and have shown early bone fixation around the artificial joint.[1] These therapeutic methods will likely become popular in the near future.

There are some patients who require these new procedures but whose MSCs cannot proliferate sufficiently or lack osteogenic potential. In these patients, allogeneic MSC transplantation will likely be needed with concomitant use of immunosuppressant drugs. In previous studies, immunosuppressant drugs have been reported to affect bone mineral metabolism in vitro[2–4] and in vivo.[5–10] However, little is known about the effect of these immunosuppressant drugs on bone marrow-derived MSCs. In particular, it is not known if any immunosuppressant drug stimulates osteoblastic differentiation in MSCs. If the most effective drug could be identified, it could be used in patients needing allogeneic MSC transplantation for bone regeneration.

This report assesses the effects of three immunosuppressant drugs, rapamycin, FK506, and cyclosporine A, on osteogenic differentiation of rat MSCs in vitro. Furthermore, we evaluate how immunosuppressant drugs modulate the effect of dexamethasone (Dex) given as an osteogenic supplement.

Materials and methods

Preparation and culture of marrow cells

The experimental protocol described in this study was approved by the animal experimentation committee of our institute. Rat bone marrow cells were prepared according to the method previously reported.[11] The femora were excised aseptically from 7-week-old male Fisher 344 rats, and both ends of the femora were cut at the epiphyses. The bone marrow was flushed out using 10 ml of culture medium expelled from a syringe through a 20-gauge needle. The released cells were collected in two T-75 flasks (Corning, Corning, NY, USA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Sigma, St. Louis, MO, USA). Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

After 10 days of primary culture, marrow stromal cells were detached from the flask using 0.25% trypsin and seeded on a six-well plate (Falcon, Franklin Lakes, NJ, USA) at 10^4 cells/cm^2 for subculture. The cells were subcultured in 2 ml of the standard medium supplemented with 80 µg/ml vitamin C phosphate (L-ascorbic acid phosphate magnesium salt n-hydrate; Wako, Osaka, Japan), 10 mM Na-β-glycerophosphate (Merck Japan, Tokyo, Japan) and with or without 10^{-8} M Dex. The cells were further cultured with one of three immunosuppressants (10^{-8} M rapamycin, 10^{-8} M cyclosporine A, or 10^{-8} M FK506) to examine whether these drugs have an osteogenic effect on MSCs. Rapamycin was purchased from BIOMOL (Plymouth Meeting, PA, USA), and cyclosporine A was purchased from ALEXIS (San Diego, CA, USA). FK506 was donated by Astellas Pharma (Tokyo, Japan). Cells were also cultured in standard medium with or without Dex to serve as controls. The medium was replaced three times a week. At weeks 1, 2, and 4, the cultures were analyzed as described below.

Analysis of cultured cell mineralization

Mineralization was determined using alizarin red S staining and phase-contrast microscopy at 1, 2, and 4 weeks after subculture. The subcultured cells were rinsed with Mosconas buffer without phosphate (0.137 M Nacl, 2.7 mM KCl, 12 mM $NaHCO_3$, and 11 mM dextrose, pH adjusted to 7.4) and stained with alizarin red S (0.25 g alizarin red S in 50 ml 0.1 M barbital buffer, pH 9) for 4 min at room temperature, then dehydrated in 70%–100% ethanol. Subcultured cells were observed with phase-contrast microscopy at weeks 1, 2, and 4 to examine cell morphology and to verify the presence of mineralized nodules.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was examined at 1, 2, and 4 weeks after subculture. The cells were washed twice in phosphate buffered saline (PBS) (Wako, Osaka, Japan) and scraped into 0.2% Nonidet-P40 (Nacalai tesque, Kyoto, Japan) containing 1 mM $MgCl_2$ (Nacalai tesque, Kyoto, Japan). The suspension was sonicated and centrifuged at 14000 g for 10 min. The supernatants (5 µl) were incubated in 1 ml of buffer containing 50 mM *p*-nitrophenylphosphate and 1 mM $MgCl_2$ at 37°C for 30 min. To stop the reaction, 2 ml of 0.2 N NaOH was added to the solution. Absorbance was measured at 410 nm to detect the *p*-nitrophenol that was released after incubation. The ALP activity of each well was expressed as micromoles of *p*-nitrophenol/30 min. To detect ALP activity in each cell, the ALP activity in each well was divided by the quantity of DNA in each well. DNA in 10 µl of suspension was stained using the DNA Quantitation Kit (Bio-Rad, Hercules, CA, USA) and bisbenzimide dye (Hoechst 33258). Fluorescence was detected, and the DNA concentration was quantitated using a fluorometer (DyNA Quant 200 Fluorometer, Amersham, Uppsala, Sweden).

Calcium assay

The calcium content was measured at 1, 2, and 4 weeks after subculture. Cell layers were scraped and sonicated as described for ALP activity. After shaking the suspension, 50 µl of the suspension was measured using the Calcium E-test (Wako, Osaka, Japan).

Osteocalcin assay

The osteocalcin content was measured at 1, 2, and 4 weeks after subculture. Cells were scraped, sonicated, and centrifuged as described for ALP activity. The supernatant was removed and the sediment was soaked in 20% formic acid for 2 weeks at 4°C to extract the osteocalcin. The extract was applied to NAP-5 columns (Amersham) and eluted with 10% formic acid to remove calcium phosphate salt. The purified extract was evaporated to collect protein fractions. The osteocalcin content was measured by doing an enzyme immunoassay using the Rat Osteocalcin EIA Kit (Biomedical Technologies, Stoughton, MA, USA).

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* test. Statistical significance was set at $P < 0.05$.

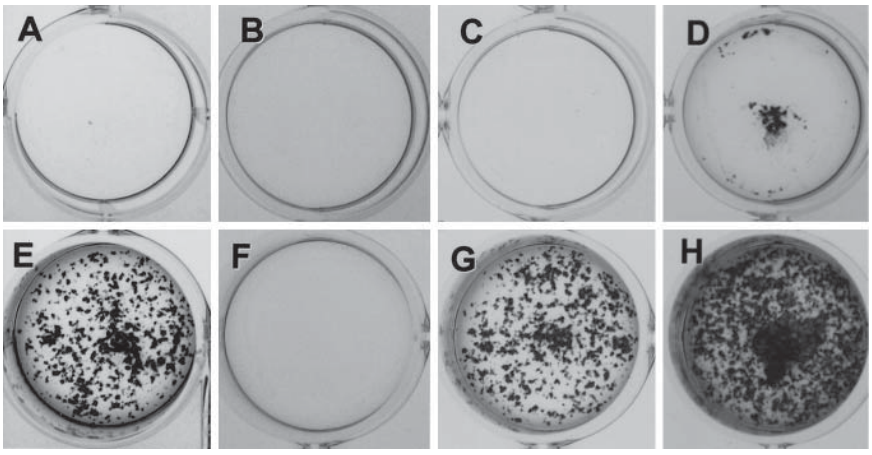

Fig. 1A–H. Alizarin red S staining of bone marrow-derived mesenchymal stem cells after 2 weeks of subculture. **A** Control without dexamethasone (Dex), **B** rapamycin without Dex, **C** cyclosporine A without Dex, **D** FK506 without Dex, **E** control with Dex, **F** rapamycin with Dex, **G** cyclosporine A with Dex, **H** FK506 with Dex

Results

Histological analysis

After 1 week of culture, mineralized nodules were not seen in any of the groups. After 2 weeks of subculture, alizarin red S-positive nodules were seen in the control group with Dex but not in the control group without Dex. In the groups without Dex, alizarin red S-positive nodules were seen only in the FK506 group; no nodules were seen in the rapamycin or cyclosporine A groups. In the groups with Dex, alizarin red S-positive nodules were seen in the cyclosporine A and FK506 groups; no nodules were seen in the rapamycin group (Fig. 1). On phase-contrast microscopy, no mineralized nodules were seen in the groups not treated with Dex, except for the group that had been treated with FK506. Mineralized nodules were seen in the groups treated with Dex, except for the group that had been treated with rapamycin (Fig. 2).

After 4 weeks of subculture, nodules were seen on alizarin red S staining and phase-control microscopy in all of the groups treated with Dex; the nodules were much fewer in the rapamycin group. In the groups not treated with Dex, nodules were seen only in the FK506 group.

ALP activity

In each group, the highest ALP activity was observed after 2 weeks of subculture. The differences in ALP activities among the groups were most significant after 2 weeks. ALP activity in the control group with Dex was significantly higher than in the control group without Dex. In the groups without Dex, ALP activity in the FK506 group was significantly higher than in the control group, but in the rapamycin and cyclosporine A groups, ALP activity was significantly lower than in the control group. In the groups with Dex, ALP activity in the rapamycin and cyclosporine A groups was significantly lower than in the control group, but in the FK506 group, ALP activity was not significantly different from that in the control group. The ALP activity in the rapamycin group was the lowest of all the groups, whether or not they were treated with Dex ($P < 0.01$) (Fig. 3).

Calcium assay

The calcium content increased in each group during the culture; the differences seen among the groups were most marked after 4 weeks of subculture: the calcium content in the control group with Dex was significantly higher than in the control group without Dex. In the groups without Dex, the calcium content in the FK506 and cyclosporine A groups was significantly higher than in the control group; the rapamycin group had a significantly lower calcium content than the control group. Among the groups without Dex, the calcium content was highest ($P < 0.01$) in the FK506 group. Among the groups with Dex, the calcium content was significantly lower in the rapamycin group than in the control group (Fig. 4).

Osteocalcin assay

In all groups, the osteocalcin content could not be detected after 1 or 2 weeks of subculture. After 4 weeks of subculture, the osteocalcin content in the control group with Dex was significantly higher than in the control group without Dex. Among the groups without Dex, the osteocalcin content in the FK506 group was significantly higher than in the control group. Among the groups with Dex, the osteocalcin content in the rapamycin group was significantly lower than in the control group (Fig. 5).

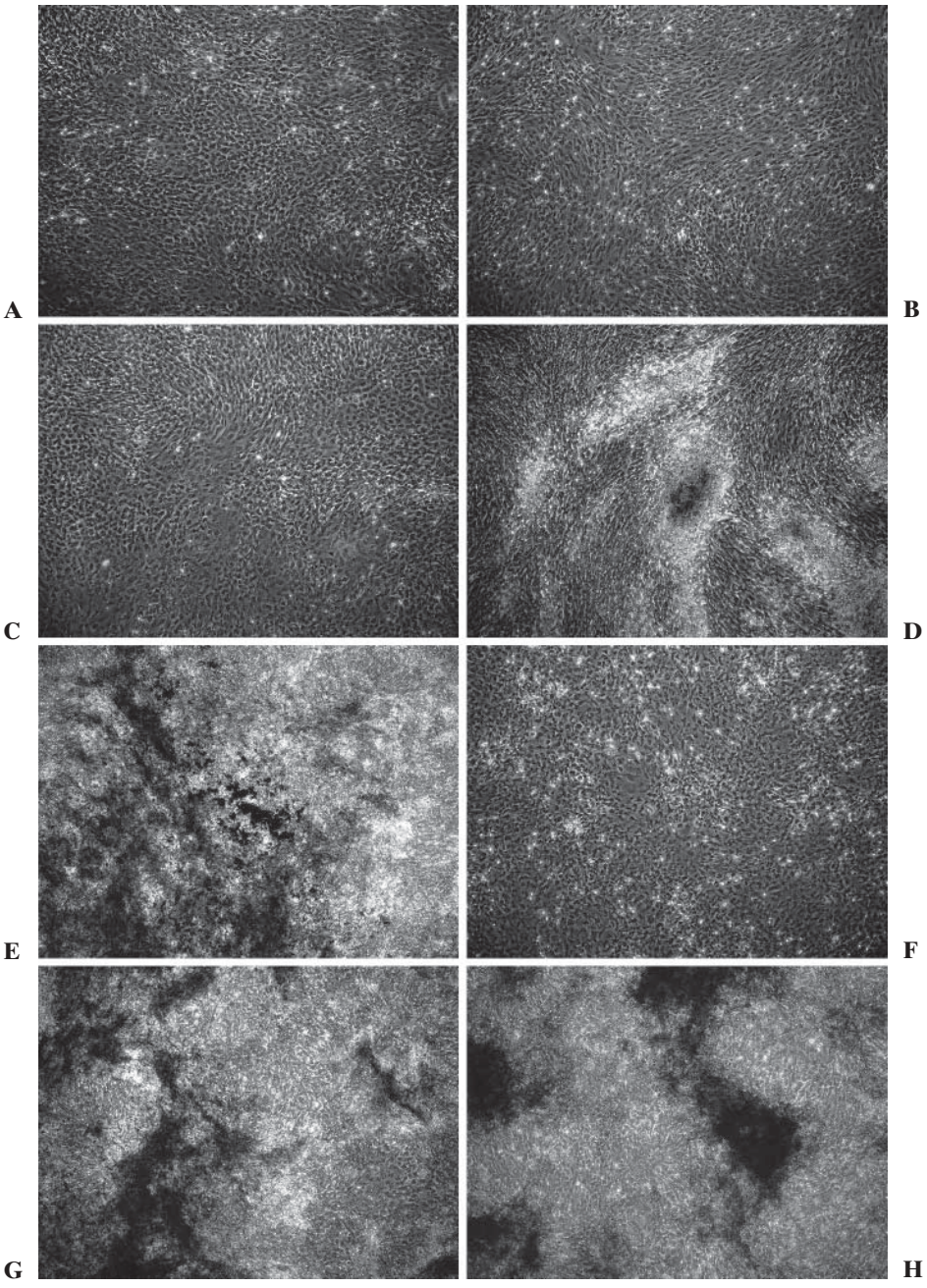

Fig. 2A–H. Phase-contrast microscopy of bone marrow-derived mesenchymal stem cells after 2 weeks of subculture. **A** Control without Dex, **B** rapamycin without Dex, **C** cyclosporine A without Dex, **D** FK506 without Dex, **E** control with Dex, **F** rapamycin with Dex, **G** cyclosporine A with Dex, **H** FK506 with Dex. ×50

Discussion

The results of this study indicate that rapamycin had no ossification-promoting or ossification-inducing effect on MSCs. Therefore, rapamycin did not have an osteogenic effect on MSCs. Moreover, based on the ALP, calcium, and osteocalcin assays, rapamycin inhibited the effect of osteogenic differentiation that was induced by dexamethasone. On the other hand, FK506 had an osteogenic effect on MSCs. Therefore, FK506 would most likely be more useful in allogeneic MSC transplantation than rapamycin.

Previously, several studies with various kinds of cells have investigated the osteogenic effect of immunosuppressants. Until now, the osteogenic effects of rapamycin have been controversial. Tang et al. reported that FK506 increased ALP activity in a mouse osteoblastic cell line and a mouse bone marrow stromal cell line.[2] They also reported that rapamycin reduced ALP activity in these cells. Shoba and Lee reported that rapamycin reduced ALP activity in rat fetal calvaria cells induced by osteogenic protein-1 and insulin-like growth factor-1.[12] These results are compatible with our results. On the other hand, Ogawa et al. reported that rapamy-

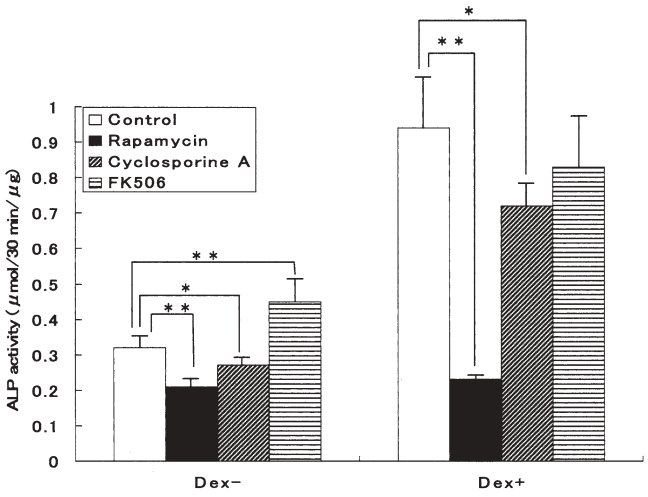

Fig. 3. Alkaline phosphatase (*ALP*) activity of cultured cells after 2 weeks of subculture. *Dex–* indicates cells cultured without dexamethasone and *Dex+* indicates cells cultured with dexamethasone. Values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$ using the Mann-Whitney *U* test

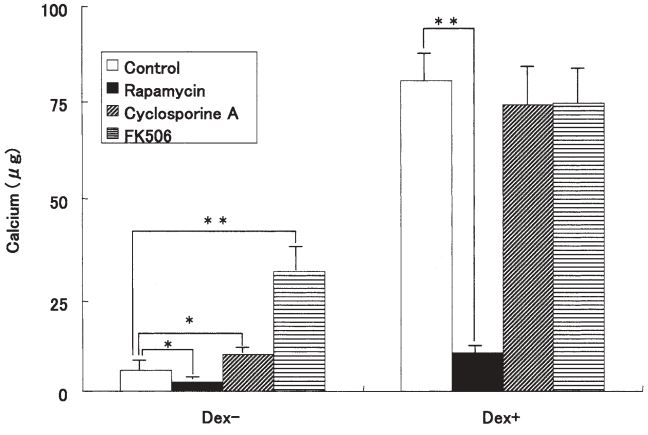

Fig. 4. Calcium content of cultured cells after 4 weeks of subculture. *Dex–* indicates cells cultured without dexamethasone and *Dex+* indicates cells cultured with dexamethasone. Values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$ using the Mann-Whitney *U* test

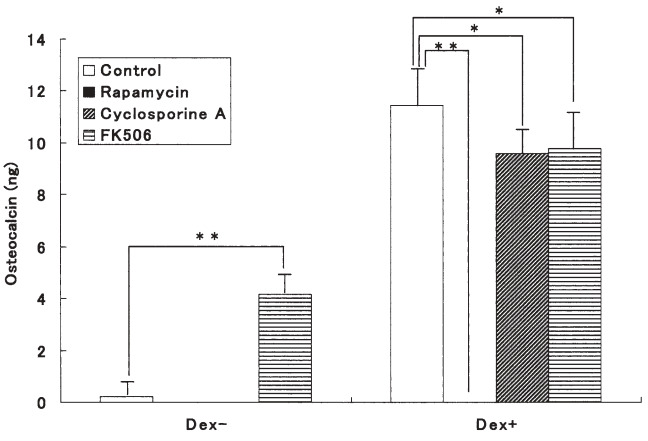

Fig. 5. Osteocalcin content of cultured cells after 4 weeks of subculture. *Dex–* indicates cells cultured without dexamethasone and *Dex+* indicates cells cultured with dexamethasone. Values represent the mean ± SD. $*P < 0.05$, $**P < 0.01$ using the Mann-Whitney *U* test

cin differentiated the immature rat osteoblast-like osteosarcoma cell line, but that FK506 and cyclosporine A had weak effects.[4] Hofbauer et al. reported that rapamycin decreased the expression of osteoprotegerin mRNA in a human marrow stromal cell line; however it increased the expression of osteoprotegerin mRNA in a human osteoblastic cell line.[13] This indicates that rapamycin has a different osteogenic effect on differentiated osteoblastic cells than on undifferentiated cells.

FK506 and rapamycin are macrolides with similar molecular structures. Rapamycin and FK506 bind to the FK506 binding protein (FKBP), but their targets on lymphocytes are different. The rapamycin/FKBP complex inhibits the activity of FKBP-rapamycin-associated protein (FRAP),[14,15] whereas the FK506/FKBP complex inhibits the activity of calcineurin.[16,17] Recent studies have indicated that FK506 and rapamycin affect the pathway that participates in osteogenic differentiation. FKBP has a specific interaction with the Transforming growth factor-β (TGF-β) type 1 receptor.[18,19] Kugiyama et al. reported that FK506 promotes osteogenic differentiation by activating the Bone morphogenetic protein (BMP) receptor through interacting with FKBP.[20] The P70 S6 kinase cascade, which is inhibited by rapamycin, has an important role in the pathway activated by the BMP family.[12,21–23] These studies can help explain how FK506 and rapamycin affect osteogenic differentiation; however, the exact mechanisms of osteogenic differentiation are still unclear, since as yet unidentified pathways may play a role.

The results of the current study provide a useful insight into the possible influence of immunosuppressants after allogeneic MSC transplantation. We previously reported that FK506 promotes bone formation in cultured MSCs implanted in vivo;[5,6] these results are in agreement with both our previous in vitro study[3] and the current study. These findings suggest that bone formation by MSCs implanted in vivo could be directly affected by FK506. We have already performed cultured MSC autografts in clinical practice.[1] However, there are some patients whose bone marrow cells cannot be used due to therapeutic restrictions. In such cases, cultured MSC allografts are a treatment option. With allografts, it is very important to select an appropriate immunosuppressant, since immunosuppressants can alter bone metabolism. The results of the current study suggest that, from the perspective of osteogenic

differentiation of MSCs, FK506 may be useful in MSC allografts, but that rapamycin may not be so useful.

In this study, we did not investigate the osteogenic effect on MSCs of the application of various concentrations of immunosuppressants. This in vitro study indicated the effects of each immunosuppressant only at a concentration of 10^{-8} M. However, we have examined the effect of rapamycin on MSCs at concentrations ranging from 10^{-9} M to 10^{-6} M (data was not shown). From these results, it was confirmed that rapamycin in concentrations ranging from 10^{-9} to 10^{-6} M did not have an osteogenic effect and inhibited the effect of osteogenic differentiation that was induced by dexamethasone. Given the promising results of this study, further studies are needed to evaluate the effects of various concentrations of immunosuppressants, which would ultimately determine the clinical usefulness of this approach.

In conclusion, rapamycin did not have an osteogenic effect on MSCs and inhibited the effect of osteogenic differentiation induced by dexamethasone. In contrast, FK506 had an osteogenic effect on MSCs. Therefore, FK506 would appear to be potentially more useful in allogeneic MSC transplantation than rapamycin.

None of the authors have financial or other relationships that might influence assessment of the data or that would constitute a conflict of interest.

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