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TAK-778 Enhances Osteoblast Differentiation of Human Bone Marrow Cells

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Abstract TAK-778 has been shown to induce bone growth in in vitro and in vivo models. However, there are no studies evaluating the effect of TAK-778 on human cells. Thus, the aim of this study was to investigate osteogenesis induced by TAK-778 on human bone marrow cells. Cells were cultured in 24-well culture plates at a cell density of 2×10^4 cells/well in culture medium containing TAK-778 (10^{-7}, 10^{-6}, and 10^{-5} M, each) or vehicle. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For attachment evaluation, cells were cultured for 4 and 24 h. After 7, 14, and 21 days, cell proliferation, cell viability, total protein content, alkaline phosphatase (ALP) activity, and bone-like formation were evaluated. Data were compared by ANOVA and Duncan's multiple range test. TAK-778 did not affect cell attachment and viability. Cell number was reduced by TAK-778 in all time period evaluated in a dose-dependent way. The effect of TAK-778 on total protein content, ALP activity and bone-like formation was a dose-dependent increase. The present results suggest that initial cell events such as cell attachment are not affected by TAK-778 while events that indicate osteoblast differentiation including reduced cell proliferation, and increased both ALP activity and bone-like formation are enhanced by TAK-778 in a time and dose-dependent way. It means that TAK-778 could be a useful drug to enhance new bone formation in clinical situations that require rapid restoration of physiologic function, such as orthopedic and maxillofacial surgery. J. Cell. Biochem. 89: 1148–1153, 2003.

Key words: cell culture; cellular differentiation; alkaline phosphatase; osteogenesis; drug therapy

Bone represents the only organ in the body capable of complete repair without the presence of an intervening fibrous scar [Hult, 1989]. However, there are clinical situations that require enhancement of the healing to ensure the rapid restoration of physiologic function, such as orthopedic and maxillofacial surgery.

Bone repair involves a complex cascade of several cell events including cell growth and differentiation. Thus, there is a great interest in studying the factors involved in these cell events to enhance osteogenesis. Drug therapies using various osseous growth factors such as bone morphogenetic proteins (BMPs), transforming growth factor β (TGF-β), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), and platelet-derived growth factor (PDGF) regulate cell proliferation, differentiation, and extracellular matrix synthesis in the initiation and the development of the bone repair [Bolander, 1992]. These growth factors are polypeptides, and have problems associated with their route of administration, cost of production, and systemic and local toxicity [Oda et al., 1999]. Therefore, their clinical application may be limited.

Notoya et al. [1994] previously reported that ipriflavone (7-isopropoxy-isoflavone), a derivative of natural isoflavone isolated from alfafa (*Medicago sativa L.*), enhances the formation of bone-like nodules in rat bone marrow cell culture due to stimulation of osteoblast differentiation. This finding and structure-activity relationship studies have led to the discovery of more potent 3-benzothiepin-2-carboxyamides derivatives from ipriflavone as the lead compound [Oda et al., 1999]. Of these, TAK-778

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[(2R, 4S)-(−)-*N*(4-diethoxyphosphorylmethylphenyl)-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide] was selected for further investigation as a new drug to stimulate osteogenesis.

TAK-778 has been shown to induce bone regeneration and stimulate fracture healing in animal models [Hoshino et al., 2000, 2001]. In vitro studies have shown that TAK-778 increases alkaline phosphatase (ALP) activity, one of the markers characteristic of the osteoblast phenotype, in rat bone marrow cell culture, and enhances the action of BMP in mouse osteoblastic cell line MC3T3-E1 [Oda et al., 1999]. TAK-778 also stimulates proliferation of uncommitted mesenchymal C3H10T1/2 cells without induction of differentiation, whereas it inhibits proliferation of osteoblast-enriched rat calvarial cells accompanied by stimulation of ALP activity [Notoya et al., 1999]. In spite of these, until the present time there are no studies evaluating the effects of TAK-778 on human bone marrow cells. Thus, the aim of this study was to investigate osteogenesis induced by TAK-778 on human bone marrow cell culture, evaluating: cell attachment, cell viability, cell proliferation, total protein content, ALP activity, and bone-like formation.

MATERIALS AND METHODS

TAK-778

TAK-778 was kindly supplied by Takeda Chemical Industries (Osaka, Japan) and it was dissolved in a solution of ethanol:dimethyl sulfoxide (1:1, v/v) at concentrations of 10^{-5}, 10^{-4}, and 10^{-3} M before use, and diluted with culture medium to the designated concentrations (10^{-7}, 10^{-6}, and 10^{-5} M).

Culture of Human Bone Marrow Cells

Human bone marrow cells were obtained from healthy male donors, 18–28 year-old, under approved research protocols of the Brazilian National Committee of Ethics in Research (CONEP) for human tissue specimens, and cultured in α-MEM (Gibco–Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco), 50 μg/ml gentamicin (Gibco), 0.3 μg/ml fungizone (Gibco), 10^{-7} M dexamethasone (Sigma, St. Louis, MO), 5 μg/L ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Subconfluent cells in primary culture were harvested after treatment with 1 mM EDTA (Gibco) and 0.25% trypsin (Gibco) and the first passage was subcultured in 24-well culture plates (Falcon, Franklin Lakes, NJ) at a cell density of 2×10^4 cells/well in culture medium containing the same volume of TAK-778 (10^{-7}, 10^{-6}, and 10^{-5} M, each). Cells subcultured in medium supplemented with vehicle were used as a control. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 3 or 4 days.

Cell Attachment

For attachment evaluation, cells were cultured for 4 and 24 h. The culture medium was removed and the wells were washed three times with phosphate buffered saline (PBS–Gibco) at 37°C to eliminate unattached cells. The adherent cells were then enzymatically (1 mM EDTA and 0.25% trypsin–Gibco) released from the well and counted using a hemacytometer. Cell attachment was expressed as percentage of the initial number of cells.

Cell Proliferation

For proliferation evaluation, cells were cultured for 7, 14, and 21 days. The culture medium was removed and the wells were washed three times with PBS at 37°C. Cells were then enzymatically (1 mM EDTA and 1.3 mg/ml collagenase and 0.25% trypsin–Gibco) released from the well, and counted using a hemacytometer.

Cell Viability

For cell viability, aliquots of the same solutions used for calculating number of cells were assayed. These aliquots were incubated, for 5 min, with the same volume of 1% trypan blue (Sigma), that stains non-viable cells, and cells were counted using a hemacytometer. Cell viability was expressed as percentage of the total number of cells counted after 7, 14, and 21 days in culture, respectively.

Total Protein Content

Total protein content was calculated, after 7, 14, and 21 days in culture, according to a Lowry et al. [1951] modified method. The culture medium was removed, the wells were washed three times with PBS at 37°C and were filled with 2 ml of 0.1% sodium lauryl sulfate (Sigma). After 30 min, 1 ml of this solution from each well was mixed with 1 ml of Lowry solution (Sigma)

and left for 20 min at room temperature. After this period, it was added to 0.5 ml of the solution of phenol reagent of Folin and Ciocalteau (Sigma). This stood for 30 min at room temperature to allow color development and the absorbance was then spectrophotometrically measured (CE3021–Cecil, Cambridge, UK) at 680 nm and the total protein content was calculated from a standard curve and expressed as μg/ml. These data were normalized by the number of cells counted after 7, 14, and 21 days, respectively.

ALP Activity

ALP activity was assayed as the release of tymolphtaleine from tymolphtaleine monophosphate using a commercial kit (Labtest Diagnostica SA, MG, Brazil), and specific activity was calculated. Aliquots of the same solutions used for calculating total protein content were assayed for measuring ALP activity. Absorbance was spectrophotometrically measured at 590 nm and ALP activity was calculated from a standard measure. Results were calculated as μmol tymophtaleine/h and data were expressed as ALP activity normalized by the number of cells counted after 7, 14, and 21 days, respectively.

Bone-Like Formation

After 7, 14, and 21 days in culture, the cells were washed three times with PBS at 37°C. The attached cells were fixed in 3% glutaraldehyde (Electron Microscopy Sciences, Washington, PA) in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and processed for staining with Alizarin red S (Sigma), that stains areas rich in calcium. The specimens were evaluated using an image analyzer (Image Tool–University of Texas Health Science Centre, San Antonio, TX) and the amount of bone-like formation was calculated as a percentage of total well area.

Statistical Analysis

Data presented in this work are the result of four separate experiments in cell cultures established from four different bone marrow donors with n = 5, for each TAK-778 concentration or control, for each experiment. All data were submitted to two-way analysis of variance (ANOVA) and Duncan's multiple range test when appropriate. Differences at $P \leq 0.05$ were considered statistically significant.

RESULTS

Cell Attachment

Cell attachment was not affected by TAK-778 (ANOVA: $F = 0.36$; D.F. = 3; $P = 0.79$), but it was a time-dependent event (ANOVA: $F = 76.65$; D.F. = 1; $P = 0.0001$) being greater after 24 h in culture. Data are presented in Figure 1.

Cell Proliferation

Cell number was reduced by TAK-778 (ANOVA: $F = 41.61$; D.F. = 3; $P = 0.0001$) in the following order: 10^{-5} M < $10^{-6} = 10^{-7}$ < Control. Data are presented in Figure 2.

Cell Viability

Cell viability was affected neither by TAK-778 (ANOVA: $F = 1.41$; D.F. = 3; $P = 0.24$) nor by period of culture (ANOVA: $F = 1.91$; D.F. = 2; $P = 0.52$). Data are presented in Table I.

Total Protein Content

Total protein content was affected by TAK-778 (ANOVA: $F = 249.21$; D.F. = 3; $P = 0.0001$) in the following order: Control < 10^{-7} M = 10^{-6} M < 10^{-5} M. Synthesis of total protein was affected by period of culture (ANOVA: $F = 175.45$; D.F. = 2; $P = 0.0001$), in the following order: 7 days < 14 days = 21 days. Data are presented in Figure 3.

ALP Activity

ALP activity was affected by TAK-778 (ANOVA: $F = 142.69$; D.F. = 3; $P = 0.0001$)

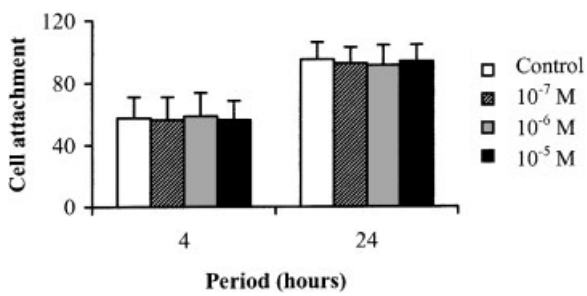

Fig. 1. Cell attachment expressed as percentage of the initial number of cells after 4 and 24 h in culture in presence of different concentrations of TAK-778. Data are reported as mean ± standard deviation (n = 5).

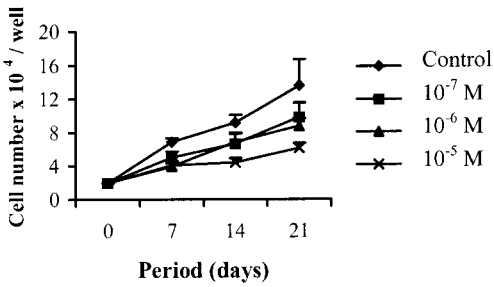

Fig. 2. Proliferation of human bone marrow cells after 7, 14, and 21 days in culture in presence of different concentrations of TAK-778. Data are reported as mean ± standard deviation (n = 5).

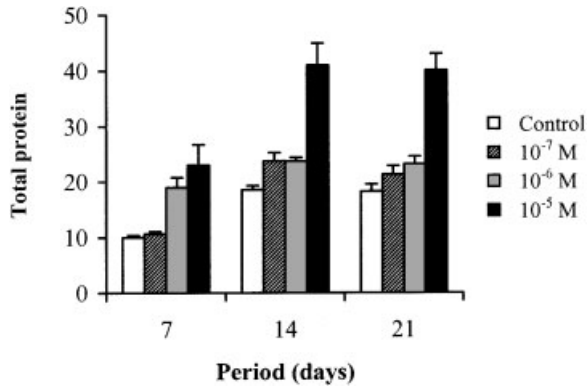

Fig. 3. Total protein content (μg/ml), normalized by the number of cells, after 7, 14, and 21 days in culture in presence of different concentrations of TAK-778. Data are reported as mean ± standard deviation (n = 5).

in the following order: Control $< 10^{-7}$ M = 10^{-6} M $< 10^{-5}$ M. It was a time-dependent event (ANOVA: F = 22.50; D.F. = 2; $P = 0.0001$) that peaked at day 14. Data are presented in Figure 4.

Bone-Like Formation

After 7 days in culture there was no evident bone-like formation (Fig. 5), so these data were not included in statistical analysis. Bone-like formation was affected by TAK-778 (ANOVA: F = 277.93; D.F. = 3; $P = 0.0001$), in the following order: Control $< 10^{-7}$ M = 10^{-6} M $< 10^{-5}$ M. Process of bone-like formation increased in a time-dependent way (ANOVA: F = 59.77; D.F. = 1; $P = 0.0001$) being greater after 21 days in culture. Data are presented in Figure 6.

DISCUSSION

Bone marrow cells are a heterogeneous population, which contain osteogenic, fibroblastic, and adipocytic cell lineages [Ozawa and Kasugai, 1996]. In this culture system, osteoprogenitor cells differentiate to osteoblasts, and osteoblasts produce extracellular matrices, which are then mineralized [Kasugai et al., 1991]. In the present study, the response of human bone marrow cells cultured in presence of different concentrations of a nonendogenous chemical compound, TAK-778, was evaluated. The results showed that TAK-778 stimulates expression of markers characteristic of the osteoblast phenotype in human bone marrow cells, which are, ALP activity and bone-like formation in a time and dose-dependent way. The in vitro concentrations of TAK-778 used in this work correspond to plasma concentrations of ipriflavone, a precursor of TAK-778, reached when it is orally administered at therapeutic doses [Yoshida et al., 1989]. Moreover, in vitro activity of TAK-778 has been reported at concentrations of 10^{-6} M or higher in rat bone marrow cells [Notoya et al., 1999].

Osteogenesis, induced by osteoblastic cells, is characterized by a sequence of events, involving cell attachment, cell proliferation and followed by the expression of osteoblast phenotype [Deligianni et al., 2001]. The initial interaction between cells and substrate produces a layer of macromolecules that modify cell behavior. Fibronectin, a cell adhesion protein present in serum, has been shown to mediate cell attachment and spread on artificial substrates by interacting with glycosaminoglycans and the cytoskeleton [Doillon et al., 1987]. This study did not show any effect of TAK-778 on cell

TABLE I. Cell Viability Expressed as Percentage of the Total Number of Cells Counted After 7, 14, and 21 Days in Culture

	Group			
Period (days)	Control	10^{-7} M	10^{-6} M	10^{-5} M
7	95.40 ± 1.88	96.21 ± 1.60	97.59 ± 2.55	95.75 ± 2.39
14	94.88 ± 2.83	94.46 ± 3.63	95.73 ± 2.97	94.93 ± 2.82
21	96.85 ± 2.49	94.17 ± 2.81	97.72 ± 0.88	95.71 ± 2.43

Data are reported as mean ± standard deviation (n = 5).

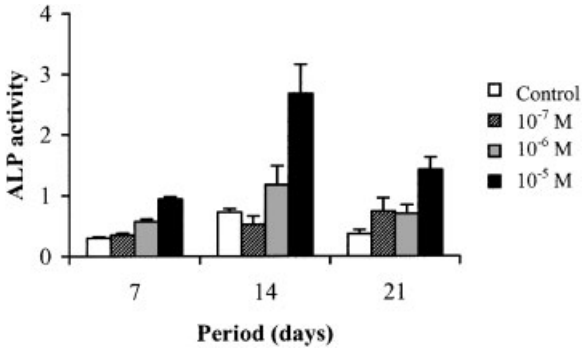

Fig. 4. Alkaline phosphatase (ALP) activity (μmol tymolphtaleine/h), normalized by the number of cells, after 7, 14, and 21 days in culture in presence of different concentrations of TAK-778. Data are reported as mean ± standard deviation (n = 5).

attachment, suggesting that TAK-778 might preferentially act on bone marrow cells at differentiation stages. However, cell attachment was a time-dependent process since there

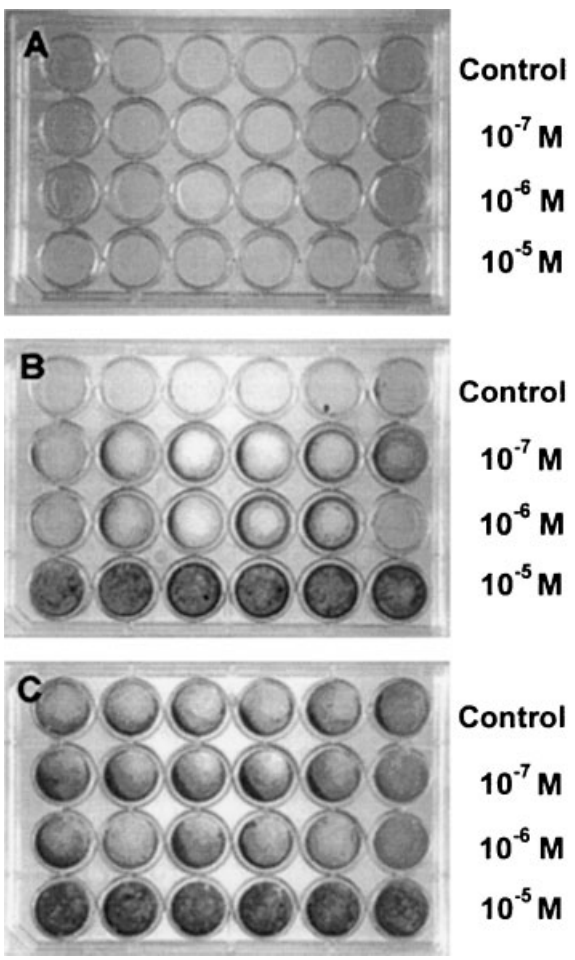

Fig. 5. Cuture plates containing bone-like formation (in dark) stained with Alizarin red after 7 days (**A**), after 14 days (**B**), and after 21 days (**C**) in culture.

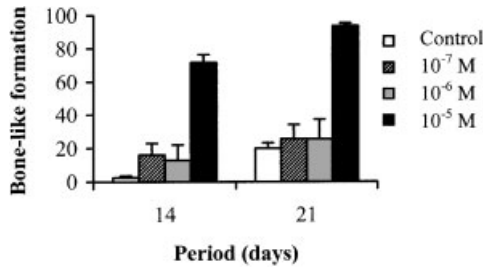

Fig. 6. Bone-like formation expressed as percentage of total well area after 14 and 21 days in culture in presence of different concentrations of TAK-778. Data are reported as mean ± standard deviation (n = 5).

were more attached cells after 24 h than after 4 h of incubation.

With regard to the relationship between cell growth and phenotypic expression during osteoblastic differentiation, Owen et al. [1999] proposed that a reciprocal relationship exists between the decrease in proliferation and the subsequent induction of cell differentiation in rat calvarial cell cultures. Therefore, the decrease in cell number caused by TAK-778, observed in this work, seems to be related to the progression of differentiation into mature osteoblasts. In agreement with these findings, Notoya et al. [1999] showed that TAK-778 at a concentration of 10^{-5} M significantly reduced the saturated cell density and increased the ALP activity of rat calvarial cells. Despite of reducing cell proliferation, TAK-778 did not affect cell viability.

TAK-778 and ipriflavone exposure result in an increase in the cell synthesis activity [Notoya et al., 1999; Hoshino et al., 2000]. The present results showed that, for human cells, TAK-778 increased the amount of total protein produced in a time and dose-dependent way. Considering that the amount of protein was normalized by the number of cells, these results evaluated the cell secretory activities that were stimulated by TAK-778.

Rat bone cells present an increase of ALP activity in presence of TAK-778, mainly at concentrations of 10^{-6} M or higher [Notoya et al., 1999]. In the present study, ALP activity could be detected in early period of culture, and peaked at day 14. It presented a dose-dependent behavior in all evaluated periods accompanied by the initiation of bone-like formation. Lincks et al. [1998] and Perizzolo et al. [2001] showed a positive correlation between ALP activity and bone-like nodule formation. In agreement with

this finding, it was possible to observe a positive correlation between ALP activity and bone-like formation in this study, and similarly to ALP activity, bone-like formation was also a dose-dependent process. Although there was no bone-like formation after 7 days, cells cultured in presence of TAK-778 at the highest concentration showed a film slightly stained by Alizarin red, suggesting that the process of bone-like formation was already occurring in a very early period of culture, what was confirmed after 14 and 21 days in culture.

The bone stimulator effect of TAK-778 has been documented in different systems of cell culture. However, this is the first study to show that TAK-778 has similar effect on cells from human tissues. The present results suggest that initial cell events such as cell attachment are not affected by TAK-778 while events that indicate osteoblast differentiation including reduced cell proliferation, and increased both ALP activity and bone-like formation are enhanced by TAK-778 in a time and dose-dependent way. It means that TAK-778 could be a useful drug to enhance new bone formation in clinical situations that require rapid restoration of physiologic function, such as orthopedic and maxillofacial surgery.

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