

Osteoblastic Differentiation of Mesenchymal Stem Cells by Mumie Extract

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ABSTRACT Mumie, a plant humus matter from rocks, is known as anabolic and a stimulator of bone regeneration in the Russian and Indian systems of health and medicine. The water-soluble fraction of mumie from Uzbekistan was characterized using ¹H NMR and infrared spectroscopic methods. The mumie extract has been investigated for its effect on osteoblastic differentiation in cell culture assays of human and murine mesenchymal stem cells. The calcium deposition and expression of alkaline phosphatase, osteocalcin, core binding factor 1 (Cbfa1), and ERK have been studied. During the 14-day assay period, human bone marrow mesenchymal stem cells (hMSCs) and human fetal osteoblasts cultured with mumie (3–5 µg/ml) underwent a dramatic change in cellular morphology, which was accompanied by a significant increase in alkaline phosphatase activity, calcium deposition, and osteocalcin expression. The expression of core binding factor 1 and ERK were enhanced in hMSCs and murine pluripotent mesenchymal precursor cell line C2C12. Dose-dependent decrease in TRAP-positive multinucleated cell formation from macrophage-like cells RAW 264.7 was observed with increasing concentration of mumie in the presence of RANKL (40 ng/ml) and PD98059 (10 µM), a specific inhibitor of ERK activity. The data suggest that mumie is a potent stimulator of osteoblastic differentiation of mesenchymal stem cells and inhibitor of osteoclastogenesis, hence it maybe of clinical benefit in the treatment for osteoporosis in human. *Drug Dev. Res.* 57: 122–133, 2002. © 2002 Wiley-Liss, Inc.

Key words: mesenchymal stem cells; osteoblasts; osteoclasts; mumie; humic substances

INTRODUCTION

Bone remodeling occurs by the action of two major cell types within bone tissue: the osteoblasts that synthesize and mineralize bone matrix, and the osteoclasts responsible for bone resorption. Osteoblasts are derived from pluripotent mesenchymal stem cells that reside within bone marrow and also give rise to adipocytes, chondrocytes, fibroblasts, and muscle cells [Pittenger et al., 1999; Jung et al., 2001]. Selective enhancement of osteoblasts activity is one of the approaches for osteoporosis therapy. An effective

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anabolic therapy would be expected to lead to a significant lowering in bone fracture risk. Investigations in the field of anabolic therapy for bone tissue are focused on fluoride, parathyroid hormone, bone morphogenic protein-2, transforming growth factor- β 1 (TGF- β 1), and several compounds (ipriflavone derivatives, statins) [Jaumberzins et al., 2000; Gowen et al., 2000]. Vegetable extracts can be regarded as a rich source of new bioactive compounds with bone remodeling activity [Muhlbauer and Li, 1999]. Ipriflavone, a derivative of isoflavone from alfalfa (*Medicago sativa* L.) stimulates rat bone marrow cells to form nodules and deposit a mineralized matrix similar to tissue bone [Notoya et al., 1994]. Plant isoflavones genistein and daidzein have a stimulatory effect on osteoblastic bone formation due to increasing protein synthesis [Yamaguchi and Sugimoto, 2000]. Among the natural products with anabolic action mumie deserves a special attention.

Mumie (common name Shilajit) has been used in the folk medicine of different countries for almost 3,000 years. In its raw form, mumie is a semihard, brownish black to dark, greasy resin that has a distinctive coniferous smell and bitter taste. In the opinion of Ghosal et al. [1991], mumie is formed due to the long-term humification of *Euphorbia* and *Trifolium* (clover) plants. Mumie can be collected throughout mountain regions in Afghanistan, Bhutan, China, Nepal, Pakistan, Tibet, Kirgizia, Tajikistan, Uzbekistan, Kazahstan, Ural, Baikal, Sayan, Caucasus, and Altai at altitudes between 1,000 and 5,000 m [Ghosal et al., 1991; Korago, 1992]. There is experimental evidence that mumie contains active chemical constituents that enhance processes of protein and nuclei acid metabolism, stimulate energy providing reactions in liver, promote the transfer of minerals, especially Ca, P, and Mg, into muscle tissue and bone, seem to possess anabolic properties, and increase muscle mass [Shvetskii and Vorobeva, 1978]. Studies of anabolic properties made it possible to use mumie in elite Russian military and sports establishment for nearly four decades for increasing strength and muscle mass as well as for its recuperative powers. Oxygenated dibenzo- α -pyrones in combination with fulvic acids were found to be the major active constituents of mumie [Ghosal et al., 1993].

Much attention was given to mumie action on bone regeneration after fractures. Mumie (0.1 g/kg, p.o., daily) caused acceleration of the primary callus formation and [32]P uptake on the focus of bone fracture in rabbits and dogs [Ismailova, 1965; Shakirov, 1965; Kelginbaev et al., 1973]. According to data obtained by Tkachenko et al. [1979], the action of mumie on bone regeneration in guinea pigs after

fracture can be different depending on the dose and time of its application after operation. The intensification of regeneration was observed when mumie was applied daily in the early periods after surgery (<7 days) at the doses of 0.26–0.30 g/kg (p.o.). Under these conditions, mumie caused twofold intensification of osteoid formation and bone mineralization. Nevertheless, the application of mumie in the same doses for 2–3 weeks after operation was accompanied by reducing the osteoid mineralization [Tkachenko et al., 1979]. Mumie (0.01 g/kg, p.o., daily) acts favorably on bone regeneration after fractures in children [Kelginbaev et al., 1973].

The available data are insufficient to understand the cellular and molecular mechanisms responsible for the anabolic and bone remodeling actions of mumie. The present study was undertaken to investigate the effects of mumie extract on osteoblastic differentiation in cell culture assays of human and murine mesenchymal stem cells. We have studied the calcium deposition and expression of osteoblastic differentiation markers such as alkaline phosphatase (ALP), osteocalcin, and core binding factor 1 (Cbfa1). ALP is known to be an early differentiation marker during bone formation [Weinreb et al., 1990]. Osteocalcin is present in bone matrix and osteoblasts and is a differentiation marker at a later stage of osteoblastic differentiation [Weinreb et al., 1990]. Cbfa1 is a new member of the Runt family of transcription factors, the expression of which is initiated in the mesenchymal condensations of developing skeleton and is strongly restricted to cells of the osteoblast lineage thereafter [Karsenty, 1998]. Cbfa1 induces expression of osteoblastic marker genes such as ALP, type I collagen, osteopontin, bone sialoprotein, and osteocalcin [Harada et al., 1999]. Cbfa1 is regulated by MAPK family members, ERK and JNK, and it is suggested that this pathway plays an important role in the control of osteogenic differentiation of mesenchymal stem cells [Jaiswal et al., 2000; Xiao et al., 2000].

Osteoclasts are multinuclear giant cells specializing in the resorption of mineral and organic components of the bone matrix. They are formed as a result of fusion of the postmitotic progenitor cells, which are ultimately derived from multipotential hemopoietic stem cells belonging to the monocyte–macrophage lineage [Takahashi et al., 1994]. Excessive activity of these cells is responsible for the bone loss that causes osteoporosis and other diseases of clinical importance [Franke and Runge, 1987]. Thus, in addition to osteoblasts formation, we have investigated the influence of mumie on osteoclastogenesis.

MATERIALS AND METHODS

Mumie Extract

Mumie was collected in the mountain region of Uzbekistan. Crude mumie powder (100 g) was shaken for 10 h at room temperature with 500 ml of distilled water, adjusted to pH 7.4, and filtered through a 0.45- μm filter. The extract was dried. For the biological experiments, the dried mumie extract was suspended in a balanced salt solution and filtrated through a 0.2- μm filter.

Spectroscopic Studies

The $^1\text{H-NMR}$ spectrum of the crude mumie extract was recorded in a 5-mm sample tube on a Bruker DRX500 high-resolution spectrometer operating at 500.13 MHz. The sample solution was prepared by dissolving approximately 100 mg of the dry mumie extract in 1 ml of D_2O , gently shaken, and filtered through glass wool into an NMR tube. Recording the spectrum of sufficient signal-to-noise ratio required 64 scans using a pulse width of 80° and recycle time of 3.7 sec.

The IR spectra were recorded with KBr pellets (2 mg of sample per 800 mg of KBr) with the use of a Specord 71 IR spectrophotometer (Carl Zeiss, Zurich, Switzerland).

Mumie Fractionation

For determining the humic substances content in mumie extract, the isolation of substances was performed by the classical method of humic substances fractionation that is based on their different solubility in water at different pH. Humic acids are defined as the class of compounds that precipitate from the alkaline humic solution upon acidification to $\text{pH} \leq 2$, whereas fulvic acids remain in solution [Stevenson, 1994]. The fractions obtained were dried and their weights were measured in percents of the total sample.

Mesenchymal Stem Cells and Osteoblasts

Human bone marrow cells were taken from female bone marrow aspirate (Hospital of Ulsan University, Korea). Human mesenchymal stem cells (hMSCs) were obtained by Ficoll density gradient fractionation (1.078 g/ml) of human bone marrow cells and cultured in a 1:1 mixture of human mesenchymal stem cell basal medium (hMSCBM) and Dulbecco modified Eagle medium (DMEM) at a concentration of 10^6 cells/ cm^2 . Nonadherent cells were removed after 5 days and then the adherent cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 . hMSC colonies were trypsinized and expanded. For confirmation of the mesenchymal nature of stem cells,

the expression of CD105 (endoglin), a receptor for transforming growth factor $\text{TGF}\beta 1$ and $\beta 3$ in bone marrow mesenchymal stem cells [Barry et al., 1999], was determined with the use of anti-mouse CD105 (cross reaction with human CD105) (Pharmingen, San Diego, CA). Anti-mouse IgG fluorescein isothiocyanate was used as a secondary antibody. After incubation for 1 h (4°C), the cells were evaluated under fluorescence-activated cell sorter (FACS) (Becton Dickinson). The results of FACS analysis show that more than 85% of cells appeared in the fibroblastic colony were the expressed CD105, which is a confirmation of mesenchymal stem cells presence in the donated bone marrow samples.

The murine pluripotent mesenchymal precursor cells of line C2C12 were maintained in DMEM containing 10% fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Human fetal osteoblasts (hFOB) cells were maintained in the 1:1 mixture of DMEM/Ham's F-12 medium containing 10% fetal calf serum and the same antibiotics. All the cells used were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. For osteoblastic differentiation (positive control) the C2C12 cells, hMSCs, and hFOB cells were cultured in osteogenic medium (Bio-Whittaker, Rockland, ME) containing dexamethasone, ascorbate, glycerophosphate, and mesenchymal cell growth factors. The cell culture medium (cell medium for negative control, osteogenic medium, and medium with mumie extract) were changed every 3 days.

ALP Activity and Histochemistry

The cells were treated with mumie extract for 14 days in 24-well plates (20,000 cells/well). After washing twice with PBS, $\text{pH} = 7.4$, the cells were scraped into 0.5 ml of PBS containing 1% Triton X-100. The cells were frozen and thawed twice, then centrifuged. An aliquot of the supernatant was used for the determination of ALP activity by measuring the release of *p*-nitrophenol from *p*-nitrophenol phosphate. Reaction was terminated by the addition of 2 M NaOH, and absorbance of the liquid was measured at 405 nm and compared with that of the reference solution. ALP activity was expressed as units/mg of protein. One unit of ALP activity hydrolyzes 1 μmol of *p*-nitrophenol phosphate per minute at 25°C , $\text{pH} 8.0$. ALP histochemical investigation was performed using Sigma Diagnostics Kit 85.

Calcium Assay

For calcium deposition assay, the cells were harvested by rinsing them in calcium-free PBS, then 0.5 N HCl was added to the well, and incubation continued for 4 h with gentle shaking. Calcium was

determined spectrophotometrically at 575 nm, that is, at the absorption maximum of the calcium-cresolphthalein complexone complex, with the use of Sigma Diagnostics kit 587. Total calcium content was calculated from standard solutions and expressed as milligrams per well.

Measurement of Osteocalcin

Osteocalcin in cell lysates was measured by enzyme-linked immunosorbent assay (ELISA) with mouse monoclonal anti-osteocalcin antibody (cross reaction with human osteocalcin) (Takara Biomedicals, Kyoto, Japan). Procedures for this experiment were followed by the standard ELISA method. Briefly, cell lysates were kept overnight under sodium bicarbonate buffer in a 96-well plate, then anti-osteocalcin OC antibody (1:1,000) reacted for 1 h at room temperature. After washing with PBS, anti-mouse ALP conjugated secondary antibody and a working substrate solution with *p*-nitrophenol phosphate were added during 35 min at room temperature. After termination of the enzymatic reaction, the stop solution was added and absorbance was measured using an ELISA plate reader with 405-nm light filter [Ahmed et al., 2000].

Cbfa1 Expression

Cbfa1 expression was confirmed by Western blotting. Briefly, the cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin and leupeptin), and lysates were frozen and thawed three times. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Richmond, CA). The cell lysates containing 25 µg of protein were separated by 12.5% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to a nitrocellulose membrane (Millipore, Marlborough, MA). The membranes were blocked with 0.1% Tween-20 in Tris buffered saline (TBS-T), pH 7.4, containing 5% dry milk for 1 h at room temperature and then incubated with anti-Cbfa1 mouse antibody (1:1,000) in 0.5% bovine serum albumin (BSA) in TBS-T at room temperature for 2 h. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibodies (1:3,000) for 1 h. After extensive washing, bands were visualized by enhanced chemiluminescence with the use of an ECL kit (Amersham Pharmacia Biotech, NJ).

ERK Expression

The cell lysates were prepared by the same procedure as in the experiments on Cbfa1 expression. The cell lysates containing 25 µg of protein were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membrane (Millipore). The membranes were blocked by 5% skim milk in TBS-T for 1 hour at room temperature and then incubated overnight with anti-ERK and phospho-ERK antibodies (1:1,000) in 0.5% BSA in TBS-T at 4°C. Anti-rabbit horseradish peroxidase-conjugated IgG (1:3,000 dilution) were used for enhanced chemiluminescence detection.

Osteoclastogenesis Assay

Murine macrophage cell line RAW 264.7 cells were maintained in DMEM containing 10% FBS, penicillin G (100 U/ml), and streptomycin (100 µg/ml). RAW 264.7 cells were plated in 24-well plates at a seeding density of 3,000 cells/well in DMEM with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. The treatment of cells was started after the cells had been incubated for 24 h at 37°C. Cells were treated with different concentrations of mumie (ranging from 200 ng/ml to 5 µg/ml) in the presence of RANKL (40 ng/ml, R & D Systems, Minneapolis, MN) and 10 µM of PD 98059 for 5 days, and the medium was changed every 3 days [Matsumoto et al., 2000; Yan et al., 2001]. Osteoclast formation was measured by counting the presence of tartrate-resistant acid phosphatase (TRAP) multinucleated positive cells (more than three nuclei) using Sigma Diagnostic Kit 387 cytochemical staining.

Statistical Analysis

Results are expressed as the mean ± SEM. Statistical comparisons were made using Student's *t*-test for paired values.

RESULTS

Spectroscopic Investigation of Mumie

¹H-NMR spectrum

The ¹H-NMR spectrum of the crude mumie extract is shown in Figure 1. The spectrum contains a set of narrow and broad signals. The narrow ones may be caused by light species coming through elaboration of material. Intense peaks at 7.3–7.5 ppm (aromatic) can be assigned to benzoic acid or its derivatives, and peaks near 1.8 ppm may arise from acetic acid. Signal at 1.2 ppm is likely to be caused by isopropanol, and peaks at 1.0 and 0.7 ppm were assigned by us to oil impurity. The broad part of the spectrum is similar to that of humus organic matter given in the literature [Wershaw, 1985; Randall et al., 1997]: 6.0–8.0: aromatic;

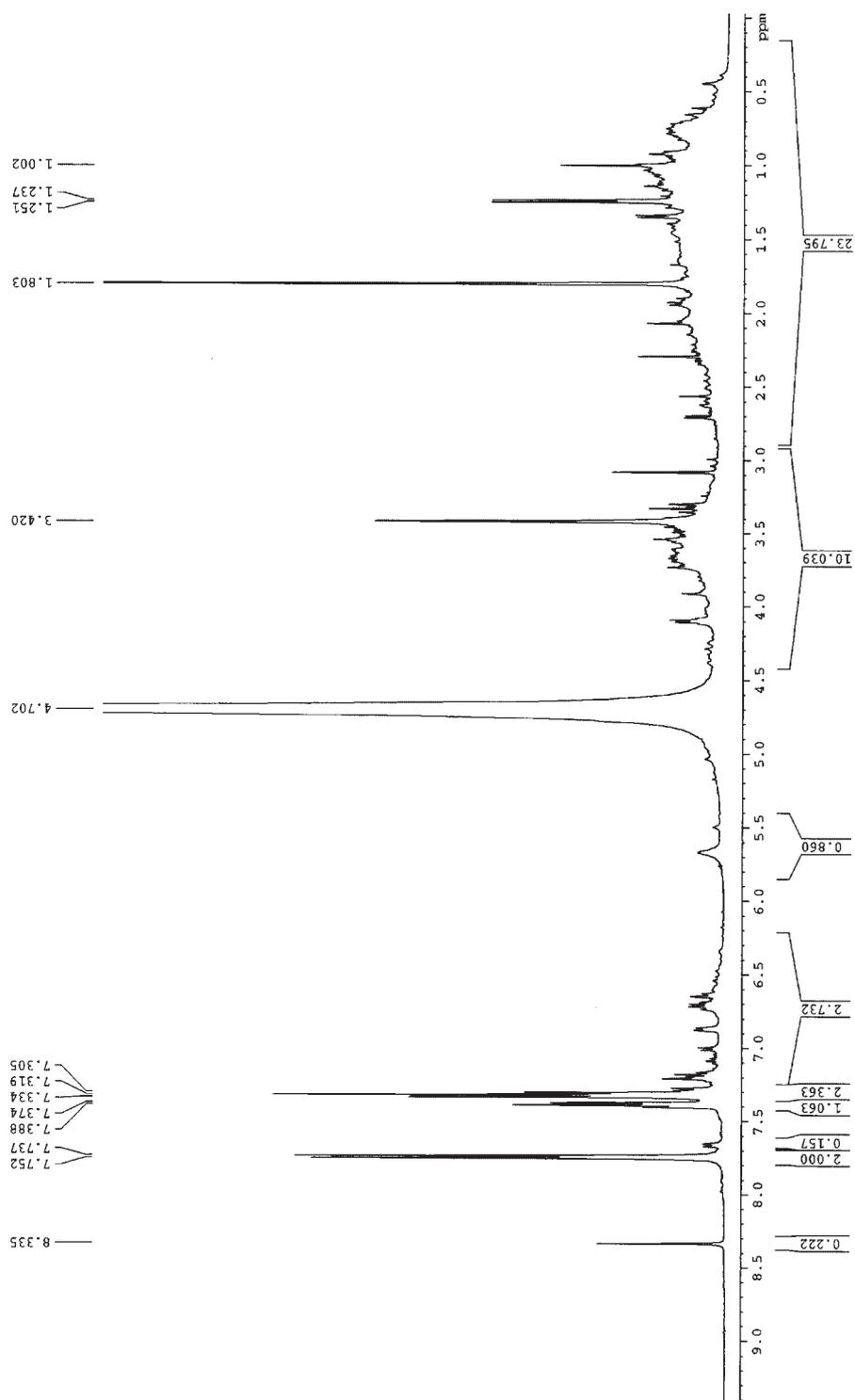


Fig. 1. ^1H NMR spectrum of the mumie extract. Spectrum was recorded on a Bruker DRX500 high-resolution spectrometer operating at 500.13 MHz.

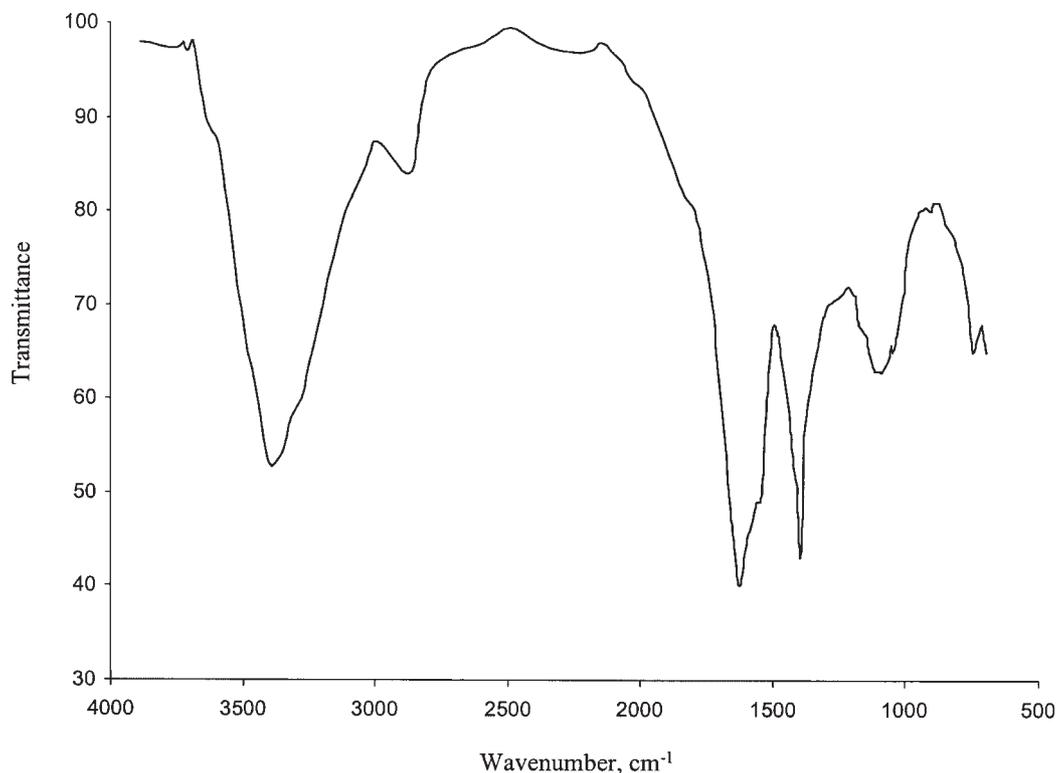


Fig. 2. Infrared (IR) spectrum of the mumie extract. The IR spectrum was recorded with KBr pellets with the use of the Specord 71 IR spectrophotometer.

~5.7 and 3.0–4.5: polysaccharides; 0.5–3.0 ppm: aliphatic signals. The strong peak at ~4.7 ppm is due to monodeuterated water formed from the deuterium exchange with the acidic protons of mumie compounds and residual protons of D₂O solvent.

IR spectrum

The characteristic absorption bands typical for humic substances are observed in IR spectrum of mumie extract (Fig. 2). The strong bands are present near 3,400 cm⁻¹ (H-bonded OH), 2,900 cm⁻¹ (aliphatic C-H stretching), 1,600–1,720 cm⁻¹ (aromatic C=C, H bonded C=O, and C=O stretching of COOH and ketonic C=O), and 1,400 cm⁻¹ (C-H bending of CH₂ or CH₃ groups).

Mumie Effect on ALP Activity

To assess the effect of mumie extract on osteoblastic differentiation of hMSCs and hFOB, cells were treated with mumie in various concentrations for 14 days and were assessed for the expression of ALP. During the cultivation period, hMSCs and hFOB cells had a dramatic change in cellular morphology accompanied by a significant increase in ALP activity ($P < 0.01$) (Fig. 3). Responsiveness of both cells was optimal when mumie was used in the concentration of

4 µg/ml. In this case, an increase in the expression of ALP achieved 35% of the level observed after the incubation of cells in the osteogenic medium. Further increase in concentration of mumie did not lead to significant increase in ALP expression.

Mumie Effect on Calcium Deposition

Mumie increased the calcium accumulation and formation of mineralized nodules in hMSCs and hFOB cells during a 14-day incubation period ($P < 0.01$). In the latter half of 14-day cultivation, the deposition of a calcified matrix on the well surface became evident by calcium staining of hFOB cell cultures. The maximal levels of calcium were 21 and 32 µg/well in hMSCs and hFOB cells, correspondingly, at 5 µg/ml concentration of mumie (Fig. 4).

Mumie Effect on Osteocalcin Production

Osteocalcin is a bone-specific protein produced by osteoblasts and is crucial in bone formation. Mumie at the concentration of 5 µg/ml significantly ($P < 0.01$) activated osteocalcin expression both in hMSCs and in hFOB cells (Fig. 5). An increase in the expression of osteocalcin reached 93.3% and 85.7% of the levels achieved by incubation of hMSCs and hFOB cells, respectively, in the osteogenic medium.

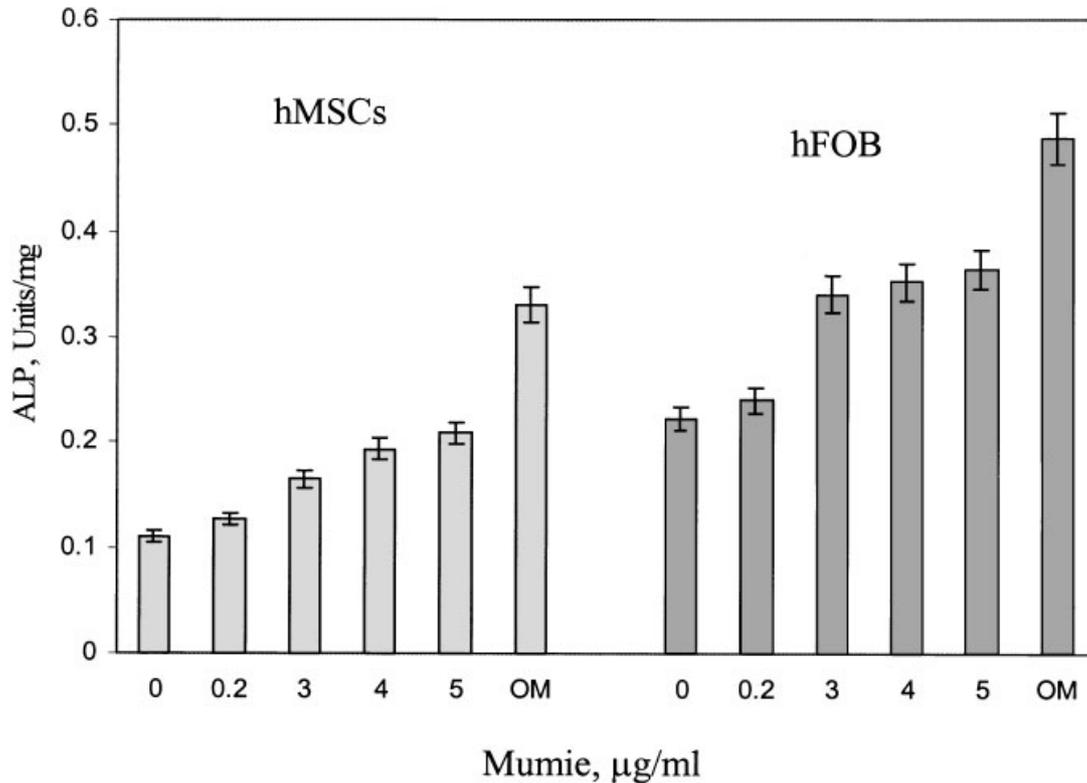


Fig. 3. Alkaline phosphatase (ALP) activity in human bone marrow mesenchymal stem cells (hMSCs) and human fetal osteoblast (hFOB) cells. hMSC and hFOB cells in a 24-well plate were cultured for 14

days with either mumie extract in Dulbecco modified Eagle medium or osteogenic medium (OM). Cell extract in 1% Triton X-100 was assayed with p-nitrophenol phosphate for ALP activity.

Mumie Effect on Cbfa1 Expression

To further address the mechanism of stimulatory effect of mumie on the osteoblastic differentiation of pluripotent stem cells, we examined protein expression of Cbfa1 and ERK in C2C12 and hMSCs. Cbfa1 is a transcription factor that regulates early phases of osteoblastic differentiation [Ducy et al., 1997; Karsenty, 1998]. Pluripotent mesenchymal precursor cell line C2C12 provides a model system to study the early events of osteoblastic differentiation during bone formation [Lee et al., 2000]. The results of our study show that mumie in concentrations from 400 ng/ml to 4 µg/ml causes the activation of Cbfa1 expression on the 7th day of incubation of C2C12 cells and hMSCs (Fig. 6).

Mumie Effect on ERK Expression

MAP kinase pathway is one of the signal transduction pathways that regulate the osteoblastic differentiation of mesenchymal stem cells [Palcy and Goltzman, 1999; Jaiswal et al., 2000]. To determine the role of ERK, a member of MAP kinase family, in mumie-induced osteoblastic differentiation, we examined the expression of ERK with anti-ERK antibody in hMSCs and C2C12 cells after 4-day incubations of the

cells with mumie. As shown in Figure 7, mumie has enhancing effect on ERK expression in hMSCs and C2C12 cells.

Mumie Effect on Osteoclast Differentiation of RAW 264.7 Cells

ERK and NF-κ B regulate different aspects of osteoclast activation: ERK is responsible for osteoclast survival, whereas NF-κB regulates osteoclast activation for bone resorption [Miyazaki et al., 2000]. Activity of NF-κB in the osteoclasts is regulated by reactive oxygen species [Hall et al., 1995]. For checking the ERK-independent effect of mumie on osteoclastic differentiation of RAW 264.7 cells, we used PD98059, a specific inhibitor of ERK activity [Matsumoto et al., 2000]. RAW 264.7 cells alone expressed high levels of RANK, but did not differentiate into TRAP-positive cells without RANKL stimulation [Huang et al., 2000]. In connection with this, we treated RAW 264.7 cells with mumie, RANKL (40 ng/ml), and PD98059 (10 µM) for 5 days and then counted the TRAP-positive cells. A dose-dependent decrease in TRAP-positive multinucleated cell formation was observed with increasing of mumie concentration (Fig. 8). These

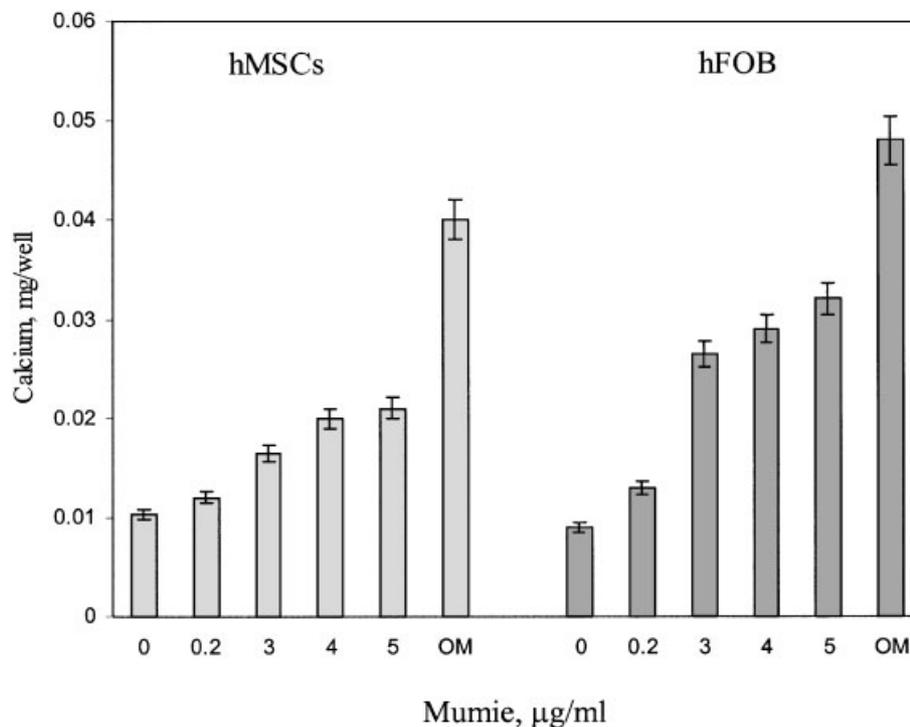


Fig. 4. Calcium accumulation in human bone marrow mesenchymal stem cells (hMSCs) and human fetal osteoblast (hFOB) cells. hMSC and hFOB cells in a 24-well plate were cultured for 14 days

with either mumie extract in Dulbecco modified Eagle medium or osteogenic medium (OM). Cell extract in 0.5 N HCl was assayed with calcium assay kit, Sigma kit No. 587.

mumie doses (from 0.2 to 5 µg/ml) were nontoxic in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) cell viability assay (data not shown).

DISCUSSION

Our studies of mumie by the $^1\text{H-NMR}$ spectroscopy confirm that the aqueous extract of mumie is composed by the complex of organic compounds of aliphatic and aromatic nature. IR spectroscopy revealed the presence of -OH, -COOH, -C=O, -CH₃ functional groups and C=C bounds of the aromatic rings. NMR and IR spectra of mumie extract are similar to the spectra of fulvic and humic acid patterns isolated from different natural sources and widely described in the literature [Wershaw, 1985; Stevenson, 1994; Shin et al., 1994; Randall et al., 1997]. Fractionation of mumie showed that the content of fulvic acids in its extract is approximately 35%. These organic acids comprise a chemically heterogeneous group of high-molecular-weight hydroxylated polyphenolic compounds with colloidal, polydispersed, and polyelectrolyte characteristics, as well as the mixture of aliphatic and aromatic moieties [Senesi and Loffredo, 1999]. Their most likely structure is a self-similar fractal, cross-linked aromatic network with different

functional groups and side chains [Cook and Landford, 1999].

Our studies showed that mumie extract in the concentrations of 4–5 µg/ml causes the activation of osteoblastic differentiation of mesenchymal stem pluripotent cells. In accordance with the levels of osteoblastic markers such as ALP activity, calcium deposition, and osteocalcin expression, the effect of mumie on osteoblastic differentiation ranges from 35% to 90% of the osteogenic medium stimulatory action. The concentrations of mumie utilized by us were close to those achievable in tissues by using mumie in clinical aspect and in experimental studies for stimulation of bone reparation after fracture [Ismailova, 1965; Kelginbaev et al., 1973]. Thus, the results obtained by us give evidence in favor of the assumption that the acceleration of bone regeneration during mumie application after fracture is connected with an increase of osteoblastic differentiation of mesenchymal stem cells. The key mechanism that causes the differentiation can be the induction of Cbfa1 and ERK. In fact, during development Cbfa1 is expressed at high levels in the cells of every mesenchymal condensation that gives rise to a skeletal element. Cbfa1 binds to the promoter of all the genes expressed predominantly in osteoblasts, such as $\alpha 1$ collagen I, bone sialo protein,

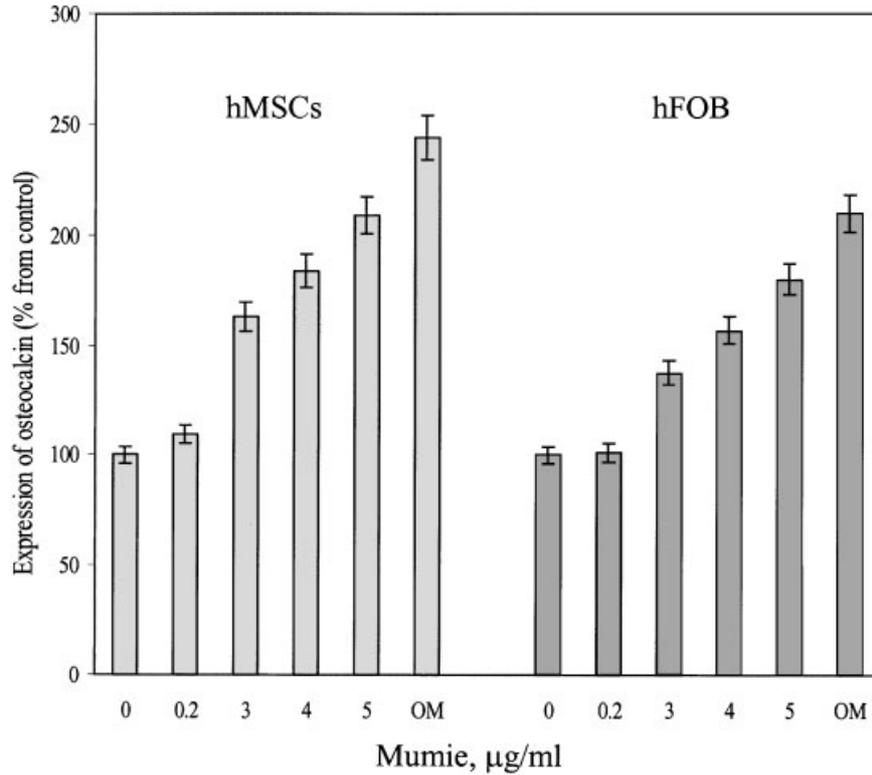


Fig. 5. Expression of osteocalcin in human mesenchymal stem cells. human bone marrow mesenchymal stem cell (hMSC) and human fetal osteoblast (hFOB) cells in a 24-well plate were cultured for 14 days with either mumie extract in Dulbecco modified Eagle medium or

osteogenic medium (OM). Cell extract in 1% Triton X-100 was assayed by enzyme-linked immunosorbent assay with anti-osteocalcin monoclonal antibody.

osteopontin and osteocalcin, and regulates positively their expression in tissue culture and in vivo [Ducy et al., 1997]. Mumie has the activation effect on ERK expression in mesenchymal stem cells during osteoblastic differentiation on the 4th day of cell cultivation. Our data are in accordance with the results of Tkachenko et al. [1979] who observed that the action of mumie on osteogenesis (in vivo) depended on dose and duration of drug application.

The literature analysis shows that mumie is a geopolymer, which can coil and folding, especially with high molecular weight species (proteins, lipids) and producing hydrophobic domains that can serve as sequestration sites for small molecules (for example, steroid nature) [Lichtfouse, 1999]. Some mumie polymers with polysaccharide nature can bind cellular receptors and activate the processes of cell differentiation. The main components of mumie, such as fulvic acids, dibenzo- α -pyrones, and ellagic acid, can act as powerful antioxidants and protect tissues from free radical damage and lipid peroxidation [Ghosal, 1989, 1995; Tripathi et al., 1996]. Fulvic acids are powerful antioxidants and possess superoxide anion (O_2^-) and hydroxyl radical scavenging properties [Wang et al.,

1996]. Taking into account that $ONOO^-$, a product of reaction between NO and O_2^- , showed a suppressive effect on osteoblastic differentiation [Hikiji et al., 2000], it is reasonable to connect the activation effect of mumie on osteoblastic differentiation of mesenchymal precursor cells with antiradical activity of mumie. Dihydroxybenzo- α -pyrones of mumie caused the reduction of dehydroascorbic acid to ascorbic acid

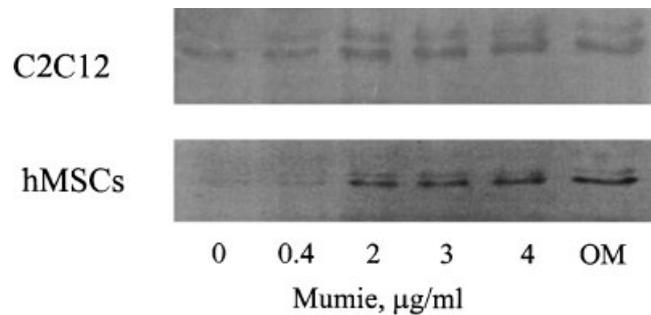


Fig. 6. Effect of mumie on core binding factor 1 (Cbfa1) expression. Mouse mesenchymal precursor cells (C2C12) or human mesenchymal stem cells (hMSCs) were incubated with indicated concentrations of mumie or in osteogenic medium (OM), and lysates were prepared on the 7th day for Cbfa1 assay as described in Materials and Methods.

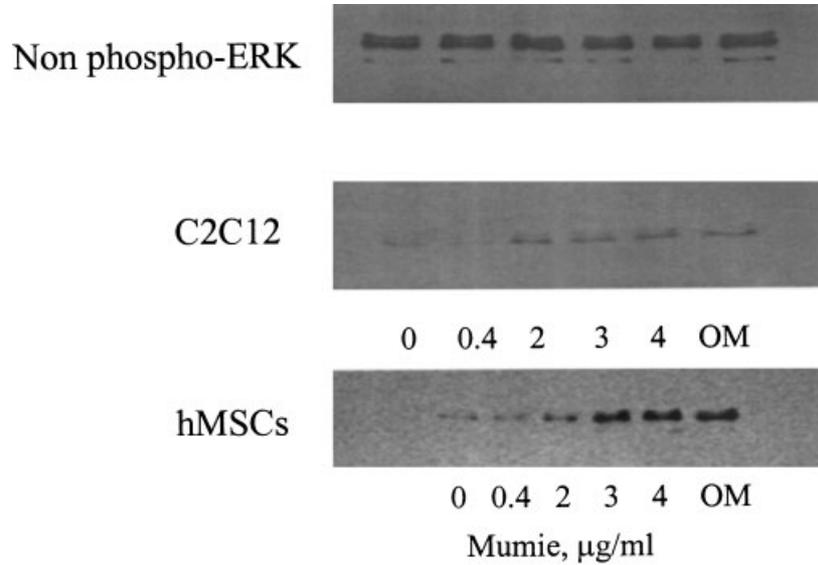


Fig. 7. Effect of mumie on ERK expression. Mouse mesenchymal precursor cells (C2C12) or human mesenchymal stem cells (hMSCs) were cultured with indicated concentrations of mumie

or in osteogenic medium (OM), and lysates were prepared on the 4th or 7th days for ERK assay as described in Materials and Methods.

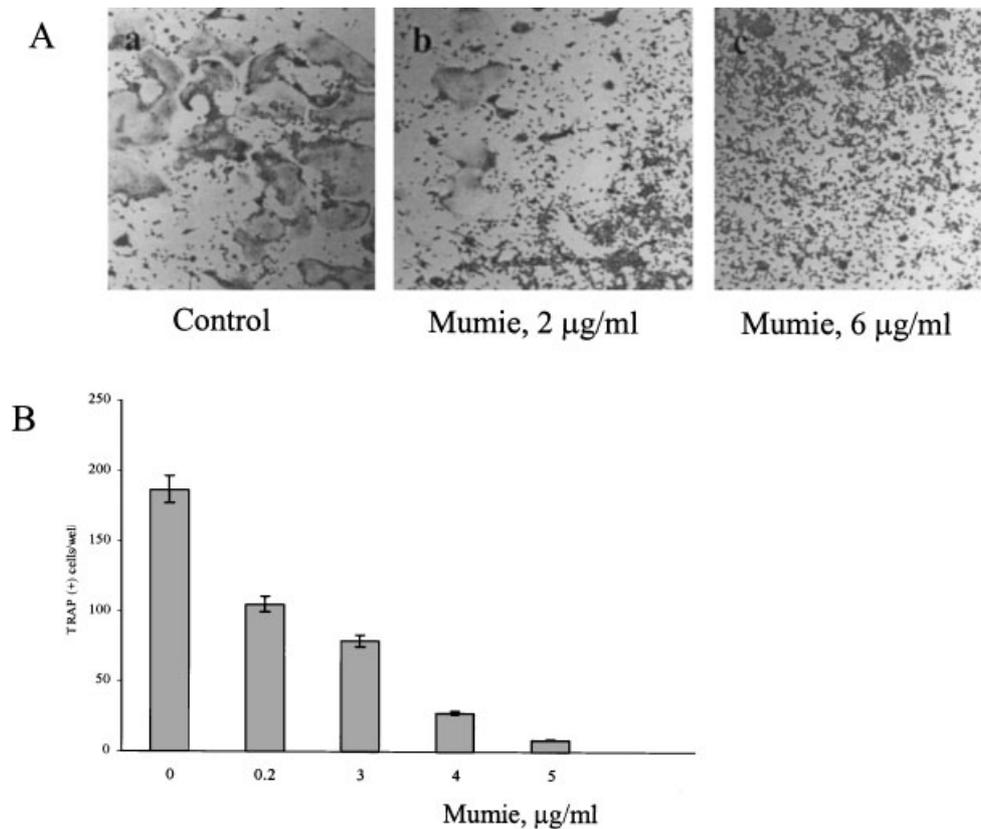


Fig. 8. Inhibition of RANKL-induced RAW 264 cell differentiation by mumie. **(A)** RAW cells were incubated for 5 days with RANKL (40 ng/ml) and PD98059 (10 mM) at varying concentration of mumie: no addition **(a)**, 2 µg/ml **(b)** and 6 µg/ml **(c)**. After incubation, cells were subjected to the TRAP assay as described in Materials and Methods.

(B) Quantitative analysis of the differentiation of RAW264 cells into TRAP-positive cells. The cells were incubated with indicated concentrations of mumie in the presence of RANKL (40 ng/ml) and PD98059 (10 mM).

[Ghosal, 1995]. In culture of bone-derived cells, ascorbic acid stimulates osteoblastic differentiation, synthesis, and deposition of collagen as well as mineralization [Sugimoto et al., 1986; Franceschi et al., 1995; Jaiswal et al., 1997; Qutob et al., 1998]. The redox state of vitamin C is important because at high concentrations dehydroascorbic acid exerts direct cytotoxic effects [Leung et al., 1993]. Furthermore, a benzopyrone compound was included in the composition of Venalot (Shaper & Brummer, Ringelheim, Germany), the drug that activates osteoblast differentiation [Lipp, 1977].

We observed the inhibitory action of mumie towards osteoclast formation. Two main pathways of regulation osteoclast activity exist: ERK- and NF- κ B-dependent ones [Miyazaki et al., 2000]. We used PD98059, a specific inhibitor of ERK activity. From the other side, the activity of NF- κ B in osteoclasts is regulated by reactive oxygen species [Hall et al., 1995]. Therefore, it is of interest to examine whether mumie constituents actually decrease the osteoclastogenesis via their anti-radical effect on NF- κ B-dependent pathway.

In conclusion, we have found that mumie is a potent stimulator of osteoblastic differentiation of mesenchymal stem cells and inhibitor of osteoclastogenesis. Further experiments are necessary to investigate the molecular mechanism of action of purified mumie constituents on osteogenesis and test their antiosteoporosis properties.

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