Behaviour of bone marrow osteoblast-like cells on mineral trioxide aggregate: morphology and expression of type I collagen and bone-related protein mRNAs

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Abstract

Nakayama A, Ogiso B, Tanabe N, Takeichi O, Matsuzaka K, Inoue T. Behaviour of bone marrow osteoblast-like cells on mineral trioxide aggregate: morphology and expression of type I collagen and bone-related protein mRNAs. *International Endodontic Journal*, **38**, 203–210, 2005.

Aim To investigate the *in vitro* behaviour of rat bone marrow cells (RBM) on mineral trioxide aggregate (MTA) (ProRoot^{TM}, MTA Root Canal Repair Material; Dentsply Tulsa, Tulsa, OK, USA) compared with intermediate restorative materials (IRM) (Dentsply Caulk, Milford, DE, USA).

Methodology RBM were obtained from rat femur and were primary cultured and then subcultured. Cells were then seeded on three dishes of each material, and cultured for 3 days, after which they were evaluated morphologically using scanning (SEM) and transmission (TEM) electron microscopy. Furthermore, the calcium released from hydrated material, the cell proliferation ratio and alkaline phosphatase (ALP) activity were analysed, and the expression of type I collagen and bone-related protein mRNAs were evaluated. The data were averaged and analysed via one-way analysis of variance (ANOVA) and were then compared by the Scheffe's test.

Results SEM showed that RBM attached to MTA and had a flattened appearance without nuclear protrusions and microspikes. TEM showed that the cells attached in the same manner as the control group, but gaps larger than 2 μm were frequently seen. The calcium released from hydrated MTA was about 130 ppm after 3 days of immersion in saline. The ALP activity was similar to the control group. Cell proliferation and expression of type I collagen mRNA was significantly lower, while the expression of osteopontin mRNA was significantly higher than the control group at the third day of culture. In IRM groups, a few rounded cells were observed on the material but no living cells were seen.

Conclusions MTA is a material of low toxicity which does not inhibit cell growth, but does suppress the differentiation of osteoblast-like cells.

Keywords: intermediate restorative material, mineral trioxide aggregate, osteoblast-like cells, rat.

Received 9 August 2004; accepted 22 October 2004

Introduction

The main objective for an apical root-end filling is to provide an apical seal that prevents the movement of bacteria and bacterial products from the root canal system into the periradicular tissues. According to Gartner & Dorn (1992), an ideal apical root-end filling material should have the following properties: easy to manipulate, radiopaque, dimensionally stable, nonabsorbable, insensitive to moisture, nontoxic and biocompatible. In addition, the root-end filling material should permit healing of the periradicular tissue in an ideal manner via regeneration of cementum, periodontal ligament, and alveolar bone across the resected

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root-end surface. Materials that have been advocated for use in root-end filling include amalgam, composite resin, zinc oxide eugenol cement, glass–ionomer cement, polycarboxylate cement and gutta-percha (Friedman 1991). Those materials are usually placed in direct contact with the periodontium for a prolonged period of time, and therefore, their biocompatibility is of primary importance.

Mineral trioxide aggregate (MTA) (ProRoot^{TM}, MTA Root Canal Repair Material; Dentsply Tulsa, Tulsa, OK, USA) was introduced not only as a new root-end filling material (Torabinejad *et al.* 1993), but also for the repair of root perforations, direct pulp capping and pulpotomy. Previous histological reports have indicated that new bone or cementum is formed adjacent to MTA when placed in contact with the periodontal tissue and the artificial bone defects (Torabinejad *et al.* 1995a, 1997, 1998, Holland *et al.* 1999, Shabahang *et al.* 1999, Moretton *et al.* 2000, Regan *et al.* 2002, Economides *et al.* 2003). Furthermore, an *in vitro* study showed that human osteosarcoma, MG-63 cells and Saos-2 cells could attach to MTA, and that MTA is a biocompatible material (Koh *et al.* 1997, 1998, Mitchell *et al.* 1999, Zhu *et al.* 2000, Pérez *et al.* 2003). Periodontal ligament cells are the major cells involved in wound healing after root-end resection (Zhu *et al.* 2000). Hypothetically, periodontal ligament cells should adhere to the MTA with matrix proteins, differentiate into cementoblasts, and create cementum on the exposed dentine and MTA. This dynamic process might play a critical role in wound healing and has implications for the growth, proliferation and differentiation of the cells (Pérez *et al.* 2003). However, little is known about the detailed events that occur on the MTA. The purpose of this study was to investigate the behaviour of rat bone marrow cells (RBM) on MTA in terms of morphology, calcium release and expression of mRNAs encoding type I collagen, ALP and bone-related proteins.

Materials and methods

Measurement of ions released from MTA

Nine millilitres of sterilized saline, pH 7.2, was added to three 60-mm cell culture discs (Corning, Tokyo, Japan) to which MTA was mixed and spread in 10 mm diameter, and then stored in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C for 3 days. Calcium, phosphorus, aluminium, silica and bismuth contents were then measured using an inductively coupled plasma optical emission spectrometer (ICP-OES; Vista-MPX, Seiko Instruments Inc., Chiba, Japan). Then, the averages of three dishes were analysed about each ion.

Cell culture and pH measurement of the culture medium

Experiments were performed according to the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College. RBM were obtained from femurs of male Wistar ST rats, weighing appropriate 100 g each (Sankyo Labo Service, Tokyo, Japan), according to the method of Maniatopoulos *et al.* (1988). The cells were then incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C for 7 days in primary culture, using α-minimal essential medium (α-MEM) (Gibco, Carlsbad, CA, USA) containing 10% foetal calf serum (Sigma, St Louis, MO, USA), supplemented with 50 mg mL^{-1} freshly prepared ascorbic acid (WAKO, Tokyo, Japan), 10 mmol L^{-1} Na β-glycerophosphate (Sigma), 10^{-8} mol L^{-1} dexamethasone (WAKO) and 10 mg mL^{-1} gentamicin (Sigma). Intermediate restorative material (IRM) and MTA were mixed and spread in the 35 mm cell culture dishes (Corning) in 10 mm diameters. Culture dishes without materials were used as controls. Three dishes of each material were used for the experiments. Cells at a concentration of 1.0×10^5 per dish on each material were seeded and cultured for 3 days. The culture medium was not changed throughout the experimental time period.

At 1, 2 and 3 days after the culture, the pH of the culture medium in each group was measured using a twin pH meter (B-212; HORIBA, Kyoto, Japan).

Morphological observations

The cultured RBM were observed every day using a phase contrast microscope. For SEM observations, cells were fixed with 2% glutaraldehyde for 1 h at 4 °C after 1, 2 and 3 days of culture. The cells were then dehydrated in an ethanol series, dried by tetramethylsilane (Merck KGaA, Darmstadt, Germany) and sputter-coated with Au-Pd (Bio-Rad, Tokyo, Japan). The cells were observed using an SEM (JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV.

For TEM observations, fixation was done in the same manner described for SEM, and post-fixed with 2% OsO_4 for 1 h. The cells were then dehydrated using an ethanol series before being embedded in Epon 812, and ultrathin sections perpendicular to the dishes were cut using an ultramicrotome (Leica Mikrosysteme GmbH,

Vienna, Austria) with a diamond knife (DiATOME, Biel, Switzerland). Sections were collected on formvar-coated copper grids and stained with saturated uranyl acetate and lead citrate. Specimens were observed using a TEM (Hitachi, Tokyo, Japan).

Cell proliferation assay

Cells were rinsed with PBS (Gibco) and then detached using 0.1% trypsin/0.02% ethylenediaminetetraacetic acid, pH 7.2. After 1, 2 and 3 days of culture, the number of detached cells was counted using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA). Data were averaged from three dishes in each group and analysed via one-way ANOVA and were then compared by the Scheffe's test.

ALP activity assay

Cells were harvested with demineralized H_2O (Milli Q, Millipore, Billerica, MA, USA), sonicated for 10 min and centrifuged at 2400 × g for 10 min. After 1, 2 and 3 days of culture, 100 μL of each cell lysate was added to 100 μL 5 mmol L^{-1} *p*-nitrophenol phosphate (Sigma) in 96-well culture plates (NUNC, Roskilde, Denmark) and incubated for 1 h at 37 °C. Reactions were stopped using 100 μL 0.3 mol L^{-1} sodium hydroxide, and the absorbance was read at 405 nm using a microplate reader (Bio-Rad). To determine the specific activity of ALP, protein concentrations in each lysate were determined using the Pierce BCA protein assay (Pierce, Rockford, IL, USA). A volume of 100 μL of each cell lysate was added to 100 μL BCA working reagent in a 96-well culture plate and incubated for 30 min at 37 °C. Absorbance was measured at 595 nm using the microplate reader. The ALP-specific activity was determined using the following formula:

$$\text{ALP specific activity}(\mu\text{mol}\,\mu\text{g}^{-1}\,\text{s}^{-1}) = \frac{\text{ALP concentration}\ (\mu\text{mol}\,\text{mL}^{-1})}{\text{Protein concentration}\ (\mu\text{g}\,\text{mL}^{-1})} \times \frac{1}{\text{ALP incubation time (s)}}$$

The averages of three dishes were analysed in each group.

Quantitative reverse transcriptase-polymerase chain reaction

Cells were detached and homogenized using TRIZOL (Invitrogen, Carlsbad, CA, USA). After 1, 2 and 3 days of culture, total RNAs were extracted. Suspensions to which chloroform was added were centrifuged at 16 000 × g for 20 min. The supernatants were recovered and mixed with isopropyl alcohol, then frozen at −80 °C overnight. Subsequently, the samples were centrifuged at 13 200 rpm for 20 min and pellets of total RNA were produced and washed with 75% ethanol. Finally, the total RNA pellets were dissolved in RNAase-free water, and stored at −80 °C until used. The total RNA was measured using a UVmini-1240 spectrometer (Shimadzu Corp., Kyoto, Japan). Using the RNA as a template, RT reactions were conducted with a reverse transcriptase-polymerase chain reaction (RT-PCR) kit (RNA-PCR kit version 2.1; Takara Biomedicals, Shiga, Japan) to synthesize cDNA. Quantitative PCR was then conducted using specific primers for: type I collagen, alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, with a LightCycler® using the double-stranded DNA dye SYBER Green I (Roche Diagnostics, Tokyo, Japan). The PCR conditions and primer sequences used in the LightCycler® are shown in Table 1. Quantification was performed by comparing the levels obtained with standardized samples. Melting curve analysis was also performed after the PCR amplification to confirm the absence of the primer dimer in the PCR products. The ratio of type I collagen, ALP, OPN and OCN mRNA expression was adjusted by the GAPDH value. Data were averaged three dishes in each group and analysed via one-way ANOVA and were then compared by the Scheffe's test.

Results

Measurement of ions released from MTA

Calcium and silica were dissolved from the hydrated MTA in saline and the release of Ca and Si were maximal at 130 and 80 ppm, respectively (Fig. 1). However, the release of phosphorus, aluminium and bismuth were extremely low throughout the time period (data not shown).

pH of the culture medium of MTA

The pH of the culture medium containing MTA remained stable at 7.5 throughout the 3-day time period (data not shown). Before the experiment, the pH of the culture medium was regulated to appropriate 7.2.

Table 1 PCR primers used for LightCycler®-assisted PCR analysis

Target cDNA	Primer sequence (5′ to 3′)	PCR condition	Product size (bp)
Type I collagen	Forward: CAA GAC AGT CAT CGA ATA CA Reverse: AGT CCA TGT GAA ATT GTC TC	45 cycles, 95 °C (10 s), 66 °C (10 s), 72 °C (10 s)	252
Alkaline phosphatase	Forward: GGC TCT CTC CAA GAC GTA CAA C Reverse: GCG TGG TTC ACC CGA GTG GT	45 cycles, 95 °C (10 s), 66 °C (10 s), 72 °C (10 s)	230
Osteopontin	Forward: CTC GGA GGA GAA GGC GCA TTA Reverse: CCA TCG TCA TCG TCG TCG TCA	45 cycles, 95 °C (10 s), 60 °C (10 s), 72 °C (12 s)	207
Osteocalcin	Forward: GGT GCA AAG CCC AGC GAC TCT Reverse: GGA AGC CAA TGT GGT CCG CTA	45 cycles, 95 °C (10 s), 60 °C (10 s), 72 °C (12 s)	199
GAPDH	Forward: TGA ACG GGA AGC TCA CTG G Reverse: TCC ACC ACC CTG TTG CTG TA	40 cycles, 95 °C (10 s), 60 °C (10 s), 72 °C (12 s)	307

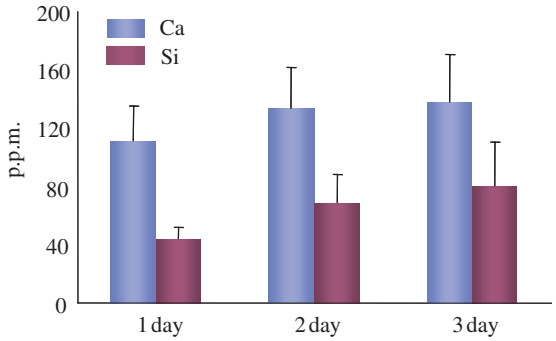

Figure 1 Measurement of ions released from MTA. Calcium and silica were dissolved from the hydrated MTA day by day.

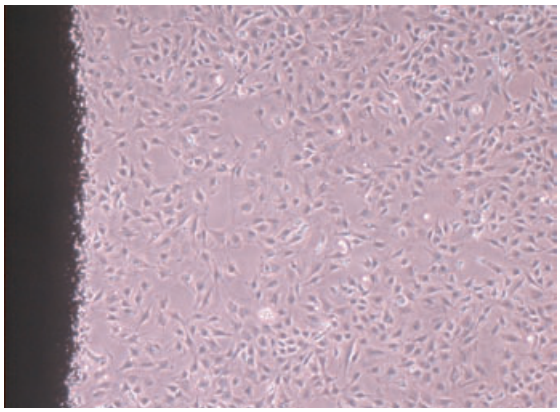

Figure 2 Phase contrast micrograph of RBM around MTA. Spindle-shaped cells were observed diffusely in the 35 mm culture dish and a few cells were attached to the rim of MTA (original magnification, ×40).

Phase contrast microscopy

In the control groups, spindle-shaped fibroblast-like cells had proliferated in the 35 mm culture dishes. In the IRM groups, almost all cells had rounded up and were floating in the culture medium and no cells were attached to the rim of material. In the MTA groups, spindle-shaped fibroblast-like cells were observed diffusely in the dish and a few cells were attached to the rim of MTA (Fig. 2).

SEM observations

In the control groups, well-spread spindle-shaped cells with many micro-spikes were observed (Fig. 3). In the IRM groups, a few rounded cells were observed on the material but no living cells were seen, and we did not do further experiments using IRM. Cells with spread and flattened cytoplasm without micro-spikes or nuclear protrusions in the cytoplasm were observed on the MTA with various porosities (Fig. 4).

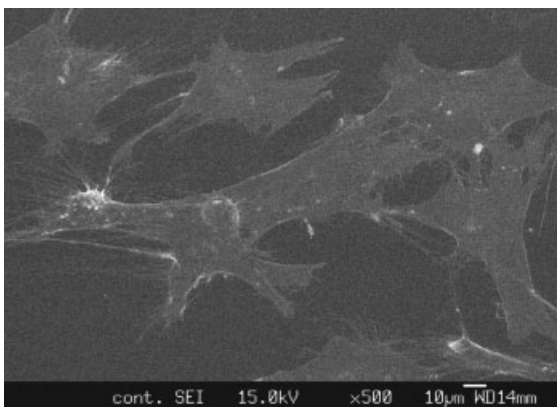

Figure 3 SEM of RBM in cell culture dishes. Cells with spreading and flattening cytoplasm were observed with micro-spikes and nuclear protrusions in the cytoplasm. These images were from day 3 of culture.

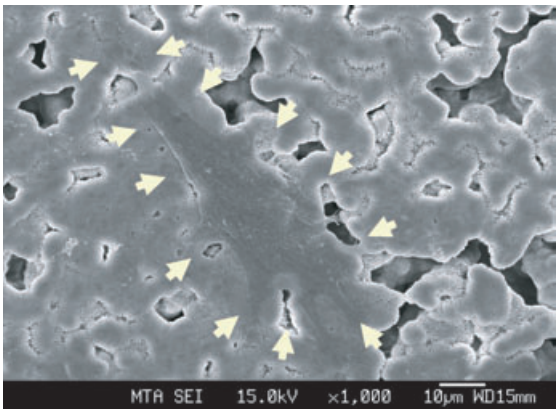

Figure 4 SEM of RBM on the MTA. Cells with spreading and flattening cytoplasm were observed. However, micro-spikes and nuclear protrusions in the cytoplasm were not observed. These images were from day 3 of culture.

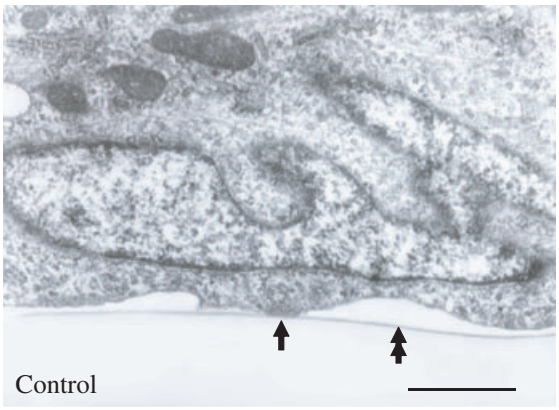

Figure 5 TEM of RBM attached to the dish with a focal contact (arrow) and ECM (double arrow). The distance between the cell and the dish is 100–300 nm (the bar indicates 1 μm).

TEM observations

In the control groups, the cell membranes were attached to the culture dishes with focal contacts, focal adhesions or ECM. The distance between the cell membrane and the culture dish was 100–300 nm in the area of the ECM (Fig. 5). In the MTA groups, well-developed endoplasmic reticulum, mitochondria and ribosomes were observed in the cytoplasm and the cell membrane was attached to the MTA in the same manner as the control groups on the flat area. Cellular bridges were created over gaps of MTA porosity (Fig. 6).

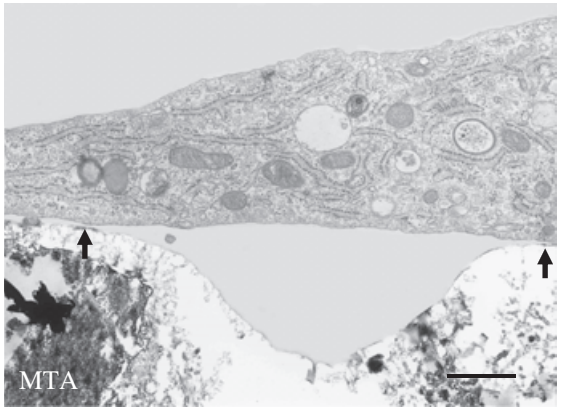

Figure 6 TEM of RBM on the MTA. The arrow shows a focal adhesion in the cell attachment (the bar indicates 1 μm).

Cell proliferation

In the control groups, cell numbers increased day by day. In the MTA groups, cell numbers increased throughout the culture period, but the rate was slower than that of the controls (Fig. 7).

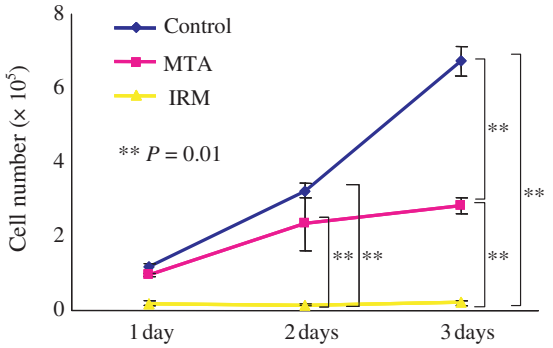

Figure 7 In the control group, the number of cells increased day by day. In the IRM group, the cells did not proliferate. In the MTA group, the cells increased in number throughout the culture period, but were lower than the control.

ALP activity

The ALP activities of the control groups increased day by day. In the MTA groups, ALP activities increased similarly to those of the control groups (Fig. 8).

Expression of type I collagen and bone-related protein mRNAs

In the MTA groups, type I collagen mRNA levels were significantly lower ($P < 0.05$) than in the control groups at each time period (Fig. 9). ALP mRNA levels

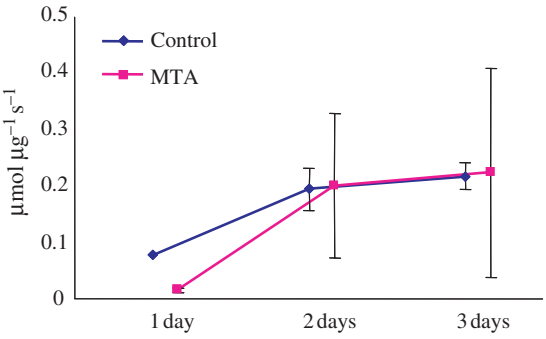

Figure 8 ALP activity of the control group increased day by day. In the IRM group, ALP activity was not detectable throughout the experimental time period. In the MTA group, ALP activity increased similarly to the control group.

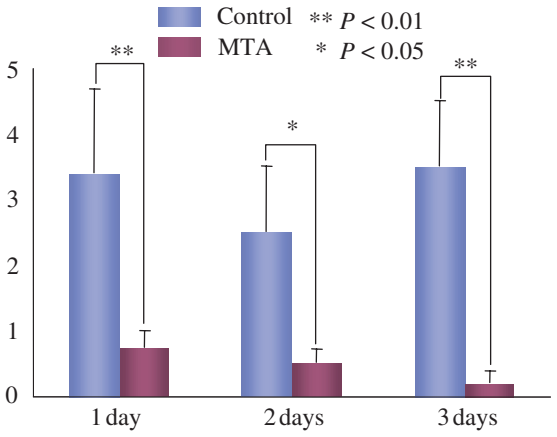

Figure 9 In the MTA group, expression of type I collagen mRNA was significantly lower than the control group at each time period.

were similar in the control groups and MTA groups throughout the experimental time period (Fig. 10). In contrast, OPN mRNA levels in the MTA groups were significantly higher ($P < 0.05$) than the control groups at each time period (Fig. 11). OCN mRNA levels decreased to almost background on both the MTA and the control groups throughout the experimental period (Fig. 12).

Discussion

MTA powder consists of fine hydrophilic particles mainly containing tricalcium silicate, tricalcium aluminate, tricalcium oxide and bismuth oxide. There are small amounts of other mineral oxides that are

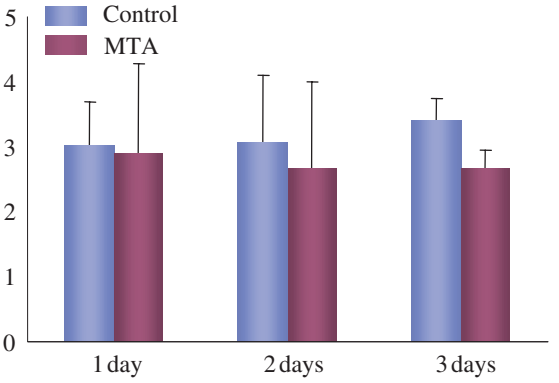

Figure 10 Expression of ALP mRNA was similar in the control group and MTA group throughout the culture periods.

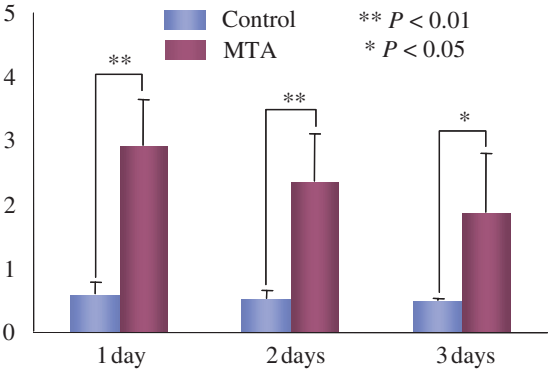

Figure 11 Expression of OPN mRNA in the MTA group was significantly higher than the control group at each time period, but did not increase.

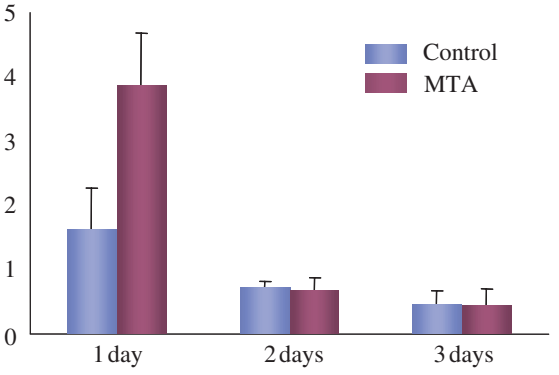

Figure 12 Expression of OCN mRNA was almost background in the MTA and control groups throughout the experimental period.

responsible for the chemical and physical properties of this aggregate (Torabinejad *et al.* 1995b). In this study, calcium release was detected at about 130 ppm

(3.25 mmol L^{-1}) from the hydrated MTA, while other elements were detected at very low concentrations. SEM and TEM analyses showed that the RBM attached to MTA in a manner similar to the control cells, but cell proliferation was not increased compared with the control groups. Orii (1999) reported that the growth, ALP activity and expression of type I collagen mRNA of the osteoblast-like cells were not significantly different between cells cultured in control medium and at low Ca^{2+} concentration (approximately 1.8–2.5 mmol L^{-1}). However, cells cultured at high Ca^{2+} concentration (approximately 10–15 mmol L^{-1}) grew slower compared with cells cultured at low Ca^{2+} concentration.

After mixing with MTA, the pH was approximately 10.2, and it increased to 12.5 at 3 h and thereafter, it remained constant (Torabinejad *et al.* 1995b). In this study, the pH of the culture medium containing MTA was stable within the range from 7.2 to 7.5 throughout the experimental time period. Taken together, there was no effect on cell growth from the Ca released from MTA or the stable pH of the culture medium. In this study, a small amount of Bi^{3+} was detected from the MTA which may have some toxicity for cell growth. For instance, bismuth is usually contained in the root canal sealer as a contrast agent. However, Yamamoto *et al.* (1998) reported that Bi^{3+} has moderate cytotoxicity against MC3T3-E1 and L-929 cells. Al^{2+} and Si^{2+} ions were also detected at low amounts, but Al^{2+} has no known cytotoxicity against cells in terms of proliferation and SiO_2 is generally a stable substance. Si might be released from MTA as SiO_2. Therefore, in terms of the small amounts of some ions released from MTA, Bi might be a toxic substance for RBM and the proliferation was less than the control groups.

OPN is related both to bone formation and to resorption, and recently Sodek *et al.* (2000) reported that OPN is also related to host defences or tissue repair. Tanabe *et al.* (2004) reported that the expression of OPN increased when osteoblasts were cultured with IL-1α which may stimulate host defences. Furthermore, Koh *et al.* (1997) reported that levels of IL-1α, IL-1β, IL-6 and M-CSF were increased when MG-63 osteoblasts were cultured in the presence of MTA, and the productions of cytokines would be modulated by the prostaglandins secreted by macrophages, known as possible mediators of inflammation. In this study, expression of OPN mRNA was significantly higher than that of the control groups, and this suggests that RBM might be protected against the toxicity through an extremely small amount of mineral oxides from MTA which contain Bi but not Ca and others described above.

Three types of contacts are known to occur between cells and matrices; focal contacts, which span less than 10 nm gaps, focal adhesions over 30–50 nm gaps (in which microfibrous bundles containing actin filaments exist in the cytoplasm), and extracellular matrix (ECM), which spans more than 100 nm gaps in which attachment proteins such as fibronectin and integrin are aggregated in the cytoplasm (Brunette 1988). TEM showed that all three kinds of cellular attachments were observed in both group, however cellular bridges over gaps in the MTA pores were frequently created with distances of over 2 μm from the gap bottom of the MTA to the cell membrane. Therefore, the proportion of the three types of cellular contacts (particularly ECM) between RBM and MTA was less than seen in the control groups. Owen *et al.* (1990) reported that type I collagen and attachment proteins are components of the ECM produced by differentiated osteoblasts. These studies suggest that RBM could be attached to the MTA but showed down-regulation of the expression of type I collagen mRNA, due to decreased development of the ECM.

It is known that OCN is secreted by osteoblasts and regulates calcification (Robey *et al.* 1972). The experimental time period in this study was up to 3 days, which is too short for RBM to differentiate into functional osteoblasts. This might be the reason why the expression of OCN was not detected in MTA groups or in control groups.

Furthermore, SEM shows that the surface of the MTA was comparatively smooth with various porosities, and that RBM attached to it spread well but micro-spikes and nuclear protrusions in the cytoplasm were not observed. In contrast, the control cells were spindle-shaped with many micro-spikes, and obvious nuclear protrusions were observed. It is known that active osteoblasts on the bone surface are cuboidal or spherical in shape, while resting osteoblasts are spindle-shaped and flat. Therefore, cells on the MTA might be similar to those in the resting phase in terms of their morphology. Taken together, we conclude that cells cultured on MTA might suppress their functions of cellular activity as osteoblastic cells but accelerate their functions as fibroblastic cells.

Conclusions

The results of this study suggest that MTA is a material with low toxicity compared with the IRM, which does

not inhibit cell growth but suppresses the differentiation of osteoblast-like cells *in vitro*.

Acknowledgements

We would like to thank to the staff of the Department of Clinical Pathophysiology, Tokyo Dental College for their helpful suggestions.

This study was supported by funding from a Grant from the Ministry of Education, Culture, Sports, Science and Technology to promote multi-disciplinary research projects, and by Grants-in-Aid for Scientific Research (C) from the Japanese Society for the Promotion of Science (No. 15592031).

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