Controlled release of simvastatin from biodegradable hydrogels promotes odontoblastic differentiation

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The objective of this study was to investigate the odontoblastic differentiation of dental pulp stem cells (DPSC) by biodegradable hydrogels incorporating simvastatin micelles, both in vitro and in vivo. Simvastatin (ST) was incorporated into the micelles of gelatin grafted with L-lactic acid oligomers (LAo) to allow water-solubilization. The simvastatin-LAo-grafted gelatin (LAo-g-gelatin) micelles were mixed with gelatin, followed by chemical crosslinking to form gelatin hydrogels (ST Mi/GH). The ST Mi were released from the gelatin hydrogel granules (GH) through enzymatic degradation. The ST Mi enhanced alkaline phosphatase activity, calcium deposition, and bone morphogenetic protein-2 secretion of DPSC. When implanted subcutaneously into mice, the ST Mi/GH treated group exhibited increased dentin sialoprotein and calcium deposition, compared with those treated with GH plus free ST. It is possible to achieve odontoblastic differentiation of DPSC through the controlled release of ST from GH.

Keywords: Controlled drug release, Dental pulp stem cells, Gelatin hydrogel, Micelle, Simvastatin

INTRODUCTION

Recently, many materials have been investigated in the design and development of pulp capping agents1-3). Among them, calcium hydroxide has been successfully used clinically and has been accepted as the pulp capping agent of first choice. Dycal® is the most commonly used drug9,14,15). In general, the DDS material should be degraded after the drug delivery has been achieved, as the in vivo persistence of delivery materials can induce inflammation and therapeutically unacceptable responses. The degradation products should also be biocompatible. Based on these characteristics, in this study, gelatin was used as the DDS material. Gelatin is a biodegradable material that has been extensively used for food, pharmaceutical, and medical purposes, and its biosafety has been proven through its use in many long-term practical applications4-6,10). Gelatin is a denatured form of collagen, which is the most abundant component of the extracellular matrix in the body tissue. Thus, the material itself and its degradation products are both biocompatible5). Additionally, ST was water-solubilized with the LAo-g-gelatin, which is commonly used to form micelles12,20,21). The objective of this study was to investigate the effect of ST Mi/GH on dentin regeneration. The ST Mi/GH was prepared to achieve controlled release of the water-insoluble ST and to evaluate the simvastatin-
induced formation of dentin in the back subcutis of mice. We also examined the in vitro induction of odontoblastic differentiation of DPSC by ST.

MATERIALS AND METHODS

Materials
Gelatin samples with isoelectric points of 5.0 and 9.0 and collagenase L were kindly supplied by Nitta Gelatin, Osaka, Japan. Disuccinimidyl carbonate (DSC) and 4-dimethylaminopyridine (DMAP) were purchased from Nacalai Tesque, Kyoto, Japan. Simvastatin, glutaraldehyde, glycine, dodecanol (DoOH), and other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan and used as obtained.

Synthesis of L-lactic acid oligomers
LаО with an average molecular weight of 1,000 were synthesized from L-lactide monomer by ring opening polymerization, with DoOH as an initiator. Briefly, 20 g of L-lactide was melted at 130°C in a nitrogen atmosphere, followed by the addition of 5.0 mL of toluene, 0.56 mL of stannous octate and 5.6 mL of DoOH. After mixing for 4 h at 130°C, the reaction product was poured into double-distilled water (DDW) for precipitation. The precipitate was then dissolved in chloroform. The solution was dropped into the DDW to obtain a white solid powder of LаО. The molecular weight of the prepared LаО was determined by 1H NMR spectroscopy (JNM-EX, JEOL, Tokyo, Japan).

Synthesis of LаО-g-gelatin
Gelatin (1.0 g; 1.0×10⁻² mol) with an isoelectric point of 5.0 was dissolved in anhydrous dimethyl sulfoxide (DMSO, 30 mL) at room temperature. The amount of LаО with a molecular weight of 1,000 (3.0×10⁻⁵ mol) was dissolved in 15 mL DMSO, and then DSC (9.0×10⁻⁵ mol) and DMAP (9.0×10⁻⁶ mol) were dissolved in 2.5 mL of DMSO, respectively. The solution was mixed to allow reaction for 3 h while stirring at room temperature to activate the hydroxyl groups of LаО. The activated LаО solution was slowly added to the gelatin solution, while the mixture was stirred overnight at room temperature for LаО grafting of the gelatin. The product solution was dialyzed against DDW with a dialysis tube with a molecular weight cut off of 12,000–14,000 for 72 h at room temperature, followed by freeze-drying to obtain the LаО-g-gelatin.

Water-solubilization of ST in LаО-g-gelatin micelles
The LаО-g-gelatin (1.0 mg/mL in DDW) and ST (2.0 mg/mL in ethanol) solutions were prepared separately. The LаО-g-gelatin solution (950 μL) and the ST solution (50 μL) were mixed, followed by stirring at room temperature for 3 h. The mixed solution was centrifuged (8,000 rpm, 10 min, 4°C) to separate the water-insoluble ST, and freeze-dried to isolate the water-solubilized ST within the LаО-g-gelatin micelles. To measure the amount of ST incorporated into the ST-LаО-g-gelatin micelles (ST Mi), the freeze-dried ST Mi was dissolved in ethanol. The solution absorbance was measured at 236 nm and the ST concentration was determined from a calibration curve prepared with ethanol containing various known amounts of ST.

Preparation of GH incorporating ST Mi
Hydrogels were prepared through chemical crosslinking of the gelatin with glutaraldehyde, according to a previously-described method. Briefly, 100 μL of an aqueous solution of gelatin with an isoelectric point of 9.0 (50 mg/mL) was added to 100 μL of the ST Mi aqueous solution (50 mg/mL). The solution was then mixed with 2.0 μL of glutaraldehyde at a concentration of 0.25 vol%, and cast in a polypropylene dish (10 mm×10 mm, Sakura Finetek Japan, Tokyo, Japan), followed by incubation at 4°C for 12 h to allow gelatin crosslinking. The cross-linked hydrogels were agitated in 100 mM aqueous glycine solution at 37°C for 1 h to block residual aldehyde groups of the glutaraldehyde. After three 1-h washes in DDW, the hydrogels were pulverized into granules using a polytron homogenizer (POLYTRON PT 3100, KINEMATICA AG, Switzerland). The GH were then freeze-dried.

To measure the concentration of ST incorporated in the GH, 5.0 mg of the GH was completely degraded by the addition of 1.0 mL of PBS solution containing 500 μg/mL of collagenase L. The resulting freeze-dried product was then dissolved in 1.0 mL ethanol and the absorbance was measured at a wavelength of 236 nm. The ST concentration was determined from a calibration curve prepared with ethanol containing various predetermined amounts of ST. The resulting products were observed with a stereomicroscope (MZ 75, LEICA, Germany) and the GH surfaces were analyzed by scanning electron microscopy (SEM; S-4000, Hitachi, Tokyo, Japan). The shapes of the GH were captured on a digital camera (EX-ZR1000, CASIO, Tokyo, Japan).

Evaluation of ST release and GH degradation
The GH incorporating ST Mi (ST Mi/GH) or GH incorporating ST (ST/GH) were placed in 1.0 mL of Dulbecco’s modified phosphate-buffered saline solution (PBS, pH=7.4) for the initial 72 h period. ST Mi/GH or ST/GH were then placed into 1.0 mL of PBS solution containing 200 μg/mL of collagenase L to allow for complete degradation. The release test was carried out at 37°C and the PBS (1.0 mL) was exchanged at specific time points. The supernatant of the collected PBS was freeze-dried and then dissolved in ethanol. After centrifugation (8,000 rpm, 10 min, 4°C), the amount of ST in the supernatant was determined according to the procedure described above. To evaluate the in vitro degradation of ST Mi/GH, the hydrogel was placed in 1.0 mL of PBS containing 200 μg/mL of collagenase L. The degradation test was performed under conditions similar to the release test. The absorbance of the PBS collected was measured at 280 nm and the gelatin concentration was determined using a calibration curve that had been generated to predetermined concentrations of gelatin and collagenase solutions.
In vitro biological evaluation of ST Mi

To evaluate the biological activity of ST Mi, an in vitro cell culture bioassay was conducted. Dental pulp stem cells (DPSC) isolated from the mandibles of C57BL/6 mice (5 weeks old, passage 3–6) were cultured at 37°C in a 95% air-5.0% CO₂ atmosphere, with changing of the medium every 3 days. After harvesting with 0.25% Trypsin-EDTA solution in PBS for 5 min at 37°C, cells were seeded into 96-well multiwell culture plates (Corning, Corning, NY, USA) at a density of 1.5×10⁴ cell/well with MEM Alpha (Prime™, Invitrogen cell culture) culture medium containing 10% fetal bovine serum (FBS) and 1.0 wt% penicillin–streptomycin. This culture medium was used for the normal medium (NM). Following 1 day of culture, the medium was replaced with MEM Alpha medium containing 10 mM β-glycerophosphate, 2.0 mM L-ascorbic acid 2-phosphate, and 100 nM dexamethasone for odontoblastic differentiation. ST/GH or ST Mi/GH were added into the differentiation medium (DM) at final concentrations of 1.0 µm. GH was used as a control. The culture supernatant was collected 7, 14, 21, and 28 days later to measure the amount of bone morphogenic protein (BMP)-2 by ELISA (Quantikine, R&D systems, Minneapolis, MN, USA). The cell lysate was also used to evaluate alkaline phosphatase (ALP) activity using the Lab Assay™ ALP (WAKO Pure Chemical Industries)., calcium content was assessed using the calcium assay (WAKO Pure Chemical Industries.), and DNA was assessed with Bisbenzimide H33258 Fluorochrome Trihydrochloride DMSO Solution (Nacalai Tesque).

In vivo evaluation of odontoblastic differentiation by ST Mi/GH

To prepare the samples for implantation, DPSC were seeded into 24-well multiwell culture plates (Corning) at a density of 3.0×10⁴ cells/well and cultured with NM for 4 days prior to implantation. After harvesting with 0.25 wt% trypsin solution in PBS for 5 min at 37°C, 1.0 mL of the cell suspension (2.0×10⁶ cells/mL) was collected into a microtube. After the suspension was centrifuged (1,000 rpm, 5 min, 4°C), the supernatant was removed and the pellet was reconstituted in medium. Next, 5.0 mg of ethylene oxide gas (EOG) sterilized GH, 0.1 and 1.0 µm of ST/GH, or 0.1 and 1.0 µm of ST Mi/GH was added to the cell suspension, followed by centrifugation (1,000 rpm, 5 min) to prepare the combination of cells and hydrogels.

An ectopic mouse model was used to evaluate the in vivo activity of ST Mi/GH. Under isoflurane anesthesia, the backs of twelve 10-week-old BALB/c immunocompromised nude mice (SHIMIZU Laboratory Supplies, Kyoto, Japan) were shaved, and disinfected. Two subcutaneous pockets were created on each flank. Each mouse received two samples from the different groups (n=4). The DPSC were mixed with GH, ST solution (ST sol), the GH mixed with 0.1µm or 1.0µm of free ST (ST/GH 0.1 or ST/GH 1.0, respectively), incorporating ST (0.1µm)-LAo-g-gelatin (ST Mi/GH 0.1), or ST (1.0 µm)-LAo-g-gelatin (ST Mi/GH 1.0) and used as samples. After implantation, the incisions were closed with staples. Animals were killed and the samples were removed 14 or 42 days after transplantation. All of the surgical procedures were performed according to protocols that were approved by the Animal Research Committee of Kyoto University.

Histological examinations

Specimens were harvested and fixed in 4% (w:v) paraformaldehyde for 24 h, and then embedded in paraffin. Three sections (4.0-µm) from around the middle of each specimen were prepared for histological analysis. The sections were deparaffinized using xylene and ethanol, and then stained with hematoxylin and eosin (HE). Von Kossa staining and immunostaining were used to visualize the calcification and evaluate the odontoblastic differentiation, respectively. For the immunohistochemical analysis, an anti-dentin sialoprotein (DSP) antibody (1:100 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used.

The areas of the calcification and the odontoblastic differentiation were quantified with NIH image analysis software (Image J).

Statistical analysis

All data were analyzed statistically by Tukey honestly significant difference test for multiple comparisons, and statistical significance was accepted at p<0.05. The experimental results are expressed as the mean±standard deviation.

RESULTS

Characterization of LAo-g-gelatin micelles and ST Mi/GH

The LAo-g-gelatin prepared in this study showed a CMC value of 79 µg/mL (data not shown), which supports the ability to form micelles. The percentage of ST water solubilization by the micelle formation was 43 wt%. Furthermore, the percentage of ST incorporated in the GH was 3.23 wt% (data not shown). The sizes of the GH and GH/Mi granules were analyzed by SEM and light-microscope images, and were found to be about 500 µm. In addition, it was apparent from the SEM image that the GH and GH/Mi granules had rough surfaces and uniformly-sized pores as a result of the freeze-drying process (Fig.1).

ST release from ST Mi/GH and GH degradation

Figure 2B shows the time profiles of ST release from ST Mi/GH in PBS with or without collagenase. An initial release of approximately 30% ST was observed in collagenase-free PBS. In the presence of collagenase, ST was released from the GH over time. Figure 2A presents the time profiles of in vitro degradation of ST Mi/GH under the same conditions. The GH did not degrade in PBS, but degraded with time after the addition of collagenase. The time profile of GH degradation was similar to that of the ST release (Figs. 2A and 2B), which indicates that the release of ST was governed by the
In vitro time profile of the degradation of ST Mi/GH (A) and the ST release (B) in PBS with or without collagenase. The ST Mi/GH was prepared at a glutaraldehyde concentration of 0.25 vol%. PBS was changed to PBS with collagenase 72 h later (indicated by the arrow).

Fig. 3  ALP activity of DPSC 7 (■), 14 (■), 21 (■), and 28 (■) days after incubation in normal (NM) and differentiation media (DM) without simvastatin. GH, ST/GH 1.0 or ST Mi/GH1.0 was cultured in DM with DPSC. *, p<0.05; significant compared with the GH group at the same time point.

degradation of GH.

In vitro biological activity of ST Mi/GH
Figure 3 shows the effect of ST and ST Mi on the ALP activity of DPSC. A significant increase in the ALP activity was observed for the ST/GH and ST Mi/GH on day 7. However, there was no significant difference in the activity between the ST/GH and ST Mi/GH groups on the other days. By contrast, the calcium content increased significantly at 21 and 28 days compared with...
Fig. 4  Ca content of DPSC 7 (□), 14 (□), 21 (□), and 28 (□) days after incubation in NM and DM without simvastatin. GH, ST/GH 1.0 or ST Mi/GH 1.0 was cultured in DM with DPSC. *, p<0.05; significant compared with the GH group at the same time point.

Fig. 5 The BMP2 secretion of DPSC 7 (□), 14 (□), 21 (□), and 28 (□) days after incubation in NM and DM without simvastatin. GH, ST/GH 1.0 or ST Mi/GH 1.0 was cultured in DM with DPSC. *, p<0.05; significant compared with the GH group at the same time point.

Fig. 6 Von Kossa staining micrographs of tissues 14 (A) and 42 days (B) after the implantation of GH (a), ST sol (b), ST/GH 0.1 (c), ST/GH 1.0 (d), ST Mi/GH 0.1 (e), ST Mi/GH 1.0 (f). The calcium deposition is shown by the black color. Original magnification ×20; scale bar=200 μm.
the other groups (Fig. 4). Significant increases in BMP-2 were observed for the ST/GH and ST Mi/GH groups at 14, 21, and 28 days (Fig. 5). ST Mi/GH exhibited biological activities in vitro at the same level as ST/GH (Figs. 3, 4, and 5).

**In vivo biological activity of ST Mi/GH**

Figures 6A and 6B show the calcification area in the histological sections with von Kossa staining. Figures 7A and 7B show the localization of DSP-positive cells in the tissues around the hydrogels 14 and 42 days after implantation. From these histological sections, the calcification area was detected within the hydrogels, and DSP-positive cells were localized within the implanted hydrogel (Figs. 6A, 6B, 7A, and 7B).

The ST Mi/GH showed the largest area of calcification at day 42 (Fig. 8). The largest number of DSP-positive cells was detected for the ST Mi/GH group at days 14 and 42 (Fig. 9). Few DSP-positive cells were observed for the GH, and ST sol groups.

**DISCUSSION**

The present study demonstrates that ST release was effective in promoting odontoblastic differentiation of DPSC. The CMC value showed that LAno-g-gelatin exhibited the ability to form micelles (data not shown). In addition, ST was able to become water-soluble with the LAno-g-gelatin. The apparent size of the ST Mi was around 200–450 nm (data not shown). The ST Mi/GH was able to release the ST in vitro through enzymatic degradation of the drug carrier hydrogels (Figs. 2A and 2B). The ST Mi exhibited biological activities to enhance ALP activity, Ca deposition and BMP-2 production from DPSC. In vivo experiments indicated that the areas of DSP-positive cells and tissue calcification were significantly enhanced by the local release of ST Mi from the ST Mi/GH.

Recently, some reports have shown that ST induces odontoblastic differentiation via the BMP-2 pathway. In addition, ST is known to induce angiogenesis and regulate the increasing neurogenesis of neuronal
The concentrations of ST used were 0.1 and 1.0 µm for the in vitro experiments, based upon previous investigations. Cellular toxicity of ST has also been reported. Therefore, in this study, toxicity testing of ST against DPSC was performed using Cell Count Reagent SF (Nakalai Tesque) (data not shown) and supported that the ST doses used in this investigation were not toxic. The in vivo experiments demonstrated that the sustained release of ST for 42 days was effective in inducing odontoblastic differentiation of DPSC in vivo.

Drug delivery systems have several advantages. Our previous study demonstrated that the release pattern of ST depended upon the extent of GH crosslinking. The extent of hydrogel crosslinking increases with an increase in the concentration of glutaraldehyde used for the hydrogel preparation. The enzymatic degradation of hydrogels become slower as the extent of crosslinking increases. A variety of hydrogels have been prepared under different conditions to investigate their properties. In this study, various hydrogels were prepared to experimentally confirm the dependence of hydrogel degradation on the extent of crosslinking (data not shown). A small surge in release was observed from the GH in PBS in which the GH was not degraded (Figs. 2A and 2B). This was due to the simple diffusion of free gelatin molecules that were not cross-linked in the hydrogels. In general, gelatin is degraded enzymatically. In the release test with collagenase, the hydrogels were degraded enzymatically to generate water-soluble gelatin fragments, resulting in the concomitant release of ST with the fragments. There was a good correlation in the time profile between ST release and the degradation of the GH (Figs. 2A and 2B). This correlation clearly indicated that the ST release was governed mainly by the degradation of GH as the release carrier. Considering the mechanism of ST release, we conclude that ST is released in a water-soluble form through incorporation into LAo-g-gelatin micelles. Furthermore, the remaining ST Mi/GH were detected 14 days after implantation. Taken together, it is conceivable that ST Mi were released from the gelatin hydrogels over the time course of 14 days, even in vivo (data not shown).

In this study, we did not investigate the effect of ST concentration on the odontoblastic differentiation. However, it is possible that the presence of ST for a longer time period is necessary to induce the cell differentiation. From Figs. 4 and 5, the BMP-2 concentration and Ca content of the ST and ST Mi groups increased significantly after 14 days of culture. From these results, the GH after 14 days degradation more strongly induced the differentiation of DPSC into odontoblasts and calcification deposition. It is possible that the duration of ST release was prolonged as the time period of degradation of GH became longer. Therefore, the GH with the longer period of degradation would release ST for a longer duration. It is possible that this sustained period of ST release prolongs the time period of in vivo bioactivity, resulting in enhanced induction of DPSC differentiation.

The micelle formation enabled ST to become water-soluble. The ST in the micelle form exhibited biological activities to induce ALP activity, calcium deposition
and BMP-2 production in vitro (Figs. 3, 4, and 5). There was no difference in the activities between ST in the free and micelle forms at the same concentrations. This clearly indicates that the activity of ST on DPSC was not impaired by the micelle formation. On the other hand, accelerated DPSC differentiation into odontoblasts was observed after stimulation with BMP for more than 10 days\textsuperscript{(10)}. When the ST Mi/GH that were degraded for 7 days were applied to DPSC, no in vitro odontoblastic differentiation was observed (data not shown), which suggests that it may be necessary to allow a controlled ST release to occur for more than 10 days. For the in vivo experiments, the percent of DSP-positive area for the ST Mi/GH was higher than that of the ST sol (Fig. 9), which indicated that the ST release from the GH was more effective for DPSC differentiation than the injection of free ST, even at the low dose. This is because ST did not remain at the injection site for a long duration. Additionally, the percent DSP-positive area for the ST Mi/GH was higher than that of the ST/GH at day 42, although both the ST Mi/GH and the ST/GH were at similar levels at day 14. This result demonstrates that free simvastatin was not be retained in the gelatin hydrogel for a long time period in vivo compared with the ST Mi. This suggests the necessity of micelle formation to prolong the simvastatin release. Similarly, the calcium deposition area for the ST Mi/GH was higher than that of ST sol (Fig. 8). The area for the GH group was also higher than that of the ST sol (Fig. 8). This suggests that the availability of GH may act as a scaffold for DPSC to enhance their activity. Additionally, as in the case with DSP-positive area, the calcium deposition area for ST Mi/GH at day 42 was higher than ST/GH. This was because ST Mi was retained in the GH for a long time period compared with free ST. Taken together, it is possible that the release of ST Mi from the GH carrier was essential for the odontoblastic differentiation of DPSC. The dose of ST necessary to induce differentiation for 42 days was 10 times lower than that of the free form (Fig. 9). This is advantageous, considering the cytotoxicity of ST at the high doses. Thus, DDS technology is one possible method to suppress drug toxicity.

In conclusion, the DDS for the release of ST is a promising method for the promotion of activity to enhance odontoblastic differentiation of DPSC and calcification. Moreover, this hydrogel system is applicable for the delivery of a wide range of water-insoluble drugs. However, further investigation is required to evaluate the in vivo time course of cell differentiation and calcification. Additionally, the quality and quantity of dentin tissue formed should be evaluated using dental pulp capping models and comparing with those of other pulp-capping materials.

CONCLUSION

ST release from the GH promoted the calcification and odontoblastic differentiation from DPSC compared with free ST, both in vitro and in vivo. In addition, these effects were observed at lower concentrations of ST. The ST Mi enhanced ALP activity, calcium deposition and BMP-2 secretion of DPSC. When implanted subcutaneously into mice, the ST Mi/GH treated group exhibited an increased tissue area of positively-stained dentin sialoprotein and calcium deposition, compared with those treated with the hydrogel plus free simvastatin. We conclude that the ST Mi/GH is a promising material to achieve direct pulp capping while minimizing drug toxicity.

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