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The effect of a single remote injection of statin-impregnated poly (lactic-*co*-glycolic acid) microspheres on osteogenesis around titanium implants in rat tibia

Tomohiro Masuzaki*, Yasunori Ayukawa, Yasuko Moriyama, Yohei Jinno, Ikiru Atsuta, Yoichiro Ogino, Kiyoshi Koyano

Section of Removable Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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ABSTRACT

The aim of this study was to evaluate the effects of newly developed injectable poly (lactic-*co*-glycolic acid) (PLGA) microspheres containing fluvastatin on osteogenesis around titanium implants in the rat tibia. After confirmation of the sustained-release profile of fluvastatin from the microspheres by an *in vitro* assay, the microspheres were administered to the back skin of the rats by a single transdermal injection. At 2 and 4 weeks after the implant surgery, the fluvastatin groups showed enhanced new bone formation around the titanium implants without any influence on the serum biochemistry. In addition, the fluvastatin groups showed increased three-point bending strengths of their femurs. The results of this study indicate that a single remote injection of PLGA/fluvastatin microspheres safely and successfully stimulated bone formation around titanium implants and increased the mechanical properties of bone.

1. Introduction

Dental implants are increasingly becoming one of the major treatment modalities for the rehabilitation of missing teeth with high predictability [1,2]. The traditional treatment protocol requires 5–6 months on average to achieve osseointegration of the implants. However, promising outcomes of immediate or early loading of dental implants have recently been reported in clinical treatment [3]. In such cases, the states of the host bone quality (density and degree of calcification) and quantity (volume) are of remarkable importance for both the initial stability and long-time outcome of the implants [4,5]. When an increase in bone quantity is desired, treatment modalities such as bone grafts, bone substitutes or certain other procedures may be introduced. However, effective methods for the improvement of bone quality have scarcely been reported. Some previous attempts to improve the bone quality of the implant site have included placement techniques, such as implant insertion into smaller dimension holes, and the use of tapered implants [6–8]. However, the effects of these techniques are expected to become clear after the accumulation of more evidence.

Statins are cholesterol-lowering drugs that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme of the mevalonate pathway [9]. However, a recent study demonstrated that statins stimulate bone morphogenetic protein (BMP)-2 expression and lead to bone formation [10]. Promotive effects of statins for the differentiation of osteoblasts have also been reported [11]. Furthermore, statins were reported to enhance bone formation in rats when administered either orally in very large doses or by once-daily or 5-day repeated injections [12]. Regarding peri-implant osteogenesis, systemic [13] or local [14] administration of statins was reported to enhance bone formation around the implants. Taking their clinical use in relation to dental implant therapy into consideration, systemic administration of statins is less preferable. However, local administration of statins has some drawbacks, including the possibility of implant surface contamination by the statin-loaded carrier and the difficulty associated with local retention of the statin-loaded carrier because of the blood flow [15].

In the present study, poly (lactic-*co*-glycolic acid) (PLGA) microspheres were selected as possible statin carriers because of their injectable and biodegradable characteristics, as well as their sustained-release profile of statins. In addition, to avoid the drawbacks of local application around the implants, we employed

* Corresponding author. Section of Removable Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6441; fax: +81 92 642 6380.
E-mail address: masuzaki@dent.kyushu-u.ac.jp (T. Masuzaki).

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remote administration into the back skin, where was far from the tibial implant site. The aim of this study was to assess the effects of a single remote percutaneous application of a statin loaded into injectable and biodegradable PLGA microspheres on osteogenesis around implants inserted into the tibias of rats. We used histomorphometry, biomechanical testing and bone marker detection to assess the effects on bone, and serum biochemistry to assess the systemic influence.

2. Materials and methods

2.1. Materials

Gelatin and PLGA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Polyvinyl alcohol and dichloromethane were purchased from Nacalai Tesque (Kyoto, Japan). Fluvastatin was obtained from Toronto Research Chemicals (North York, Canada). Sprague-Dawley (SD) rats were purchased from Kyudo (Tosu, Japan). Pure titanium implants were fabricated by Skyblue (Fukuoka, Japan).

2.2. Fabrication of fluvastatin-impregnated PLGA microspheres

Fluvastatin-impregnated PLGA microspheres were prepared using an in-water drying method reported by Ogawa et al. [16], who loaded leuprolide. Briefly, the inner water phase consisted of 450 mg of fluvastatin in a mixture of 2.5 ml of water and 400 mg of gelatin at approximately 60 °C. The oil phase consisted of 400 mg of PLGA dissolved in 5 ml of dichloromethane solution. The oil phase was gradually poured into the inner water phase under vigorous stirring with a magnetic stirrer over a few minutes to make a microfine water/oil (w/o) emulsion. The emulsion was cooled to approximately 15 °C to increase the viscosities of both the inner water phase and the w/o emulsion itself, and then poured into 400 ml of an aqueous 0.25% polyvinyl alcohol solution under vigorous stirring with a magnetic stirrer over 2 min to make a (w/o)/w emulsion. To evaporate the dichloromethane, the (w/o)/w emulsion was gently stirred with a magnetic stirrer for several days while the emulsion was warmed to approximately 30 °C. The hardened microspheres in the wet state were sized using sieves with apertures of 125, 88, 74, 44 and 37 μm, rinsed with water three times and lyophilized into powders. The powders were dried under reduced pressure for several days. We also created PLGA microspheres without the statin as controls.

2.3. Observation of microspheres

To check the microspheres, they were coated with gold (JEC-550 Twin Coater; JEOL, Tokyo, Japan) and examined with a scanning electron microscope (JEM-1210; JEOL) at 15 kV (Fig. 1).

2.4. In vitro fluvastatin release test

The cumulative release profile of fluvastatin from the microspheres was determined using a colorimetric assay [17], with some modifications. The microspheres (1 mg) were suspended in 2 ml of simulated body fluid (SBF) at 37 °C. An aliquot (70 μl) of the SBF supernatant was removed daily and analyzed by spectrophotometry.

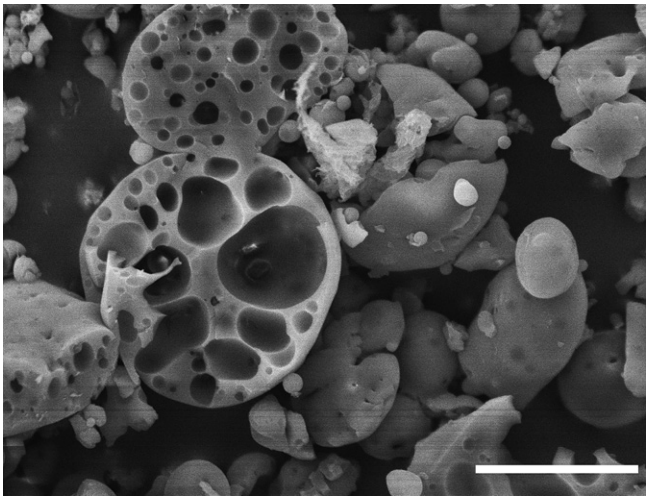

Fig. 1. SEM image of the microspheres. Bar = 20 μm.

2.5. In vivo animal experiment

2.5.1. Animals

Under approval from the Committee for Animal Research of Kyushu University, 40 female SD rats (10 weeks old; body weight, 200–220 g) were used in the present study. For this experiment, the rats were housed under identical conditions, and fed a commercially available standard food containing 1.25% calcium, 1.06% phosphate and 2 IU/g of vitamin D3 (CE-2; CLEA Japan, Tokyo, Japan). Water was available *ad libitum*.

2.5.2. Implantation

Pure titanium rods (diameter, 1 mm; length, 1.5 mm; average surface roughness, 0.5 μm) were sterilized in an autoclave before implantation. The implantation was performed under systemic anesthesia using pentobarbital sodium, which was supplemented as necessary. Each hind limb was scrubbed with alcohol and exposed, and a hole (1 mm in diameter) was created at 10 mm below the knee joint. After the cavity was flushed with sterile saline, an implant was inserted into each hole.

2.5.3. Injection of microspheres

After completion of the implant surgery, the animals were divided into four groups and received a 2-ml percutaneous injection of one of the following items at the back skin: physiological saline (passive control group); microspheres (active control group); microspheres containing 0.5 mg/kg of fluvastatin (PF0.5 group); and microspheres containing 1.0 mg/kg fluvastatin (PF1.0 group). At 2 or 4 weeks after the treatment, the animals were euthanized, and perfusion fixation was performed. For histologic analysis, the tibias were harvested without the soft tissues. Afterwards, undecalcified ground sections of approximately 70-μm thickness parallel to the long axis of the implant were fabricated, as reported previously in ref. [18].

2.5.4. Determination of peri-implant bone volume and bone-implant contact

The specimens were stained using the Masson-Goldner method. Images were obtained by light microscopy and both the percentage of new bone-titanium contact along the total length of the implant surface in the medullary canal (BCR) and the percentage of new bone in the total area of the medullary canal (BV) were calculated, as described previously [13]. These histomorphometric procedures were performed using NIH Image 1.62 (NIH, Bethesda, MD).

2.5.5. Serum biochemistry

To assess the systemic influence of a single transdermal application of statin-loaded microspheres, a serum biochemistry assessment was performed. At the time of sacrifice at 2 or 4 weeks after the implantation, a blood sample was obtained from the heart. Liver and muscle enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), a renal index (creatinine) and total cholesterol were analyzed.

2.5.6. Bone metabolism markers

Blood samples were obtained at 2 and 4 weeks after the implantation. As a bone resorption marker, tartrate-resistant acid phosphatase (TRAP) activity was analyzed using a *p*-nitrophenylphosphate method (TRACP & ALP Assay Kit; Takara Bio, Shiga, Japan). As a bone formation marker, the serum osteocalcin (OCN) level was measured using a sandwich ELISA method (Rat Osteocalcin EIA Kit; Biomedical Technologies, Stoughton, MA).

2.5.7. Biomechanical testing of femurs

At 2 or 4 weeks after the implantation, the femurs were removed and the soft tissues were removed. To evaluate the effects of fluvastatin on bones other than the tibias, the femurs were subjected to three-point bending with a Universal Testing Machine (AG-IS; Shimadzu, Kyoto, Japan). Each femur was horizontally positioned

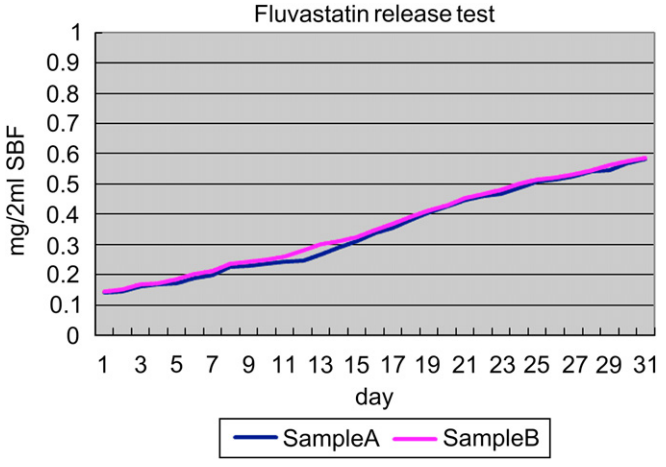

Fig. 2. Cumulative release amounts of fluvastatin.