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ORIGINAL ARTICLE

Subcutaneous administration of lactone form of simvastatin stimulates ectopic osteoinduction by rhBMP-2

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OBJECTIVE: To evaluate the effects of various 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) on ectopic osteoinduction by recombinant human bone morphogenetic protein-2 (rhBMP-2) using different administration methods.

MATERIALS AND METHODS: Disks containing 5 μg of rhBMP-2 and type I collagen were implanted into the calf muscles of 6-week-old male rats ($n = 64$). Either the lactone form of simvastatin (SV), open hydroxy-acid form of simvastatin (SVA), cerivastatin (CVA), or vehicle (control) was then administered per orally (PO group) or subcutaneously (SC group) for 20 days. The disks were removed on day 21 after implantation, and ectopic induced bone formation was evaluated by radiographic, histologic, and biochemical analysis.

RESULTS: Both the projected and radiopaque area on X-ray film, and the calcium content of the SV group in the SC group (SV–SC group) were significantly greater than those in the other SC and PO groups. Alkaline phosphatase activity and tartrate-resistant acid phosphatase activity in the SV–SC group were significantly lower than those in the other SC and PO groups. Histologic examination revealed an increase of ectopic induced bone volume in the SV–SC group.

CONCLUSION: Subcutaneous administration of SV stimulates ectopic osteoinduction by rhBMP-2 through reduction of bone turnover.

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Keywords: bone morphogenetic protein; bone turnover; cerivastatin; osteoinduction; simvastatin; statin

Introduction

Bone morphogenetic proteins (BMPs) are pluripotent growth factors that belong to the transforming growth factor beta (TGF-β) superfamily, and in particular, BMP-2 strongly induces osteoblastic differentiation of immature mesenchymal cells *in vitro* (Yamaguchi *et al*, 1991) and induces ectopic bone formation *in vivo* (Wang *et al*, 1990). Clinically, recombinant human BMP-2 (rhBMP-2) is very useful because of its bone-inductive ability, and local application of BMP-2 has been expected. In the oral and maxillofacial regions, application of BMP-2 was attempted for mandibular reconstruction after resection of malignant neoplasms, repair of bone defects as a result of cleft palate or periodontitis, and bone augmentation for dental implants. However, there is a significant need for the development of methods to enhance the bone-inducing activity of BMP-2, because a large quantity of BMP is necessary to obtain a sufficient bone volume in humans.

Statins inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme for cholesterol biosynthesis, and can reduce serum cholesterol concentration. Mundy *et al* (1999) demonstrated that statins enhance the expression of BMP-2 mRNA in osteogenic cells and stimulate bone formation in rodents. Some *in vitro* studies have indicated that statins promote differentiation of osteoblastic cells (Luckman *et al*, 1998; Sugiyama *et al*, 2000; Ohnaka *et al*, 2001; Parhami *et al*, 2002) and induce osteoclast apoptosis (Fisher *et al*, 1999) through the inhibition of the cholesterol biosynthetic pathway. In addition, several clinical studies have reported a reduction in fracture incidence (Chan *et al*, 2000; Meier *et al*, 2000; Wang *et al*, 2000; Pasco *et al*, 2002), and a significant increase in bone mineral density (BMD) associated with those patients who received oral statins (Edwards *et al*, 2000; Funkhouser *et al*, 2002).

Recently, we have reported that not only exogenous rhBMP-2, implanted in muscles of rats, but also endogenous BMP-2 produced by osteogenic cells participate in the progression of ectopic bone formation (Nakagawa *et al*, 2001). If statins really enhance the

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expression of BMP-2 mRNA, the possibility exists that they promote the production of endogenous BMP-2 and stimulate osteoinduction. In the present study, we administered statins per orally (PO) or subcutaneously (SC) to rats for 20 days after intramuscular implantation of rhBMP-2. The objective of this study was to examine the effects of statins on rhBMP-2-induced ectopic bone formation.

Materials and methods

Reagents

Recombinant human BMP-2 was generously provided by Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). RhBMP-2 was suspended in buffer (5 mM glutamic acid, 2.5% glycine, 0.5% sucrose and 0.01% Tween 80, pH 4.5) and stored at −80°C. Porcine skin-derived atelopeptide type I collagen (AC) solution (3 mg ml^{-1}, pH 3.0, Cellmatrix LA; Nitta Gelatin, Inc., Osaka, Japan) was used as the vehicle. Simvastatin was donated by Merck, Sharp & Dohme, Inc. (Rahway, NJ, USA), which was provided in the lipophilic and inactive lactone form (SV). Cerivastatin was kindly provided by Bayer, A.G. (Wuppertal, Germany), which was provided in the hydrophilic and active open hydroxy-acid form (CVA).

Preparation of statins

As it is known that SV is hydrolyzed into the active open hydroxy-acid form (SVA) after oral administration, we examined the influence of SV and SVA, respectively. SV was suspended in 0.5% sodium carboxymethylcellulose (CMC; low viscosity, Sigma-Aldrich Co., St. Louis, MD, USA) solution and the final concentration of SV was adjusted to 4 mg ml^{-1}. To convert SV into SVA, 4 mg of SV was dissolved in 100 μl of ethanol, to which 150 μl of 0.1 N NaOH was added. After heating at 50°C for 2 h, the mixture was neutralized with HCl and made up to a final volume of 1 ml with 0.5% CMC solution. CVA was dissolved in 0.5% CMC solution and the final concentration of CVA was adjusted to 0.12 mg ml^{-1}.

Implantation of rhBMP-2 collagen disks

Recombinant human BMP-2 (5 μg) was combined with 1 ml of AC solution. This mixture was lyophilized and then compressed in an injection syringe to form the disk (4 mm in diameter, 1.5 mm in thickness). Six-week-old male Wistar rats ($n = 64$, 110–130 g body weight) were anaesthetized by intraperitoneal injection of sodium pentobarbital. After shaving the skin of the left hind limb, an incision (10 mm) overlying the posterior aspect of the left calf was made. A disk consisting of rhBMP-2 and AC (BMP disk) was inserted into the left calf muscle of each rat. After implantation, the fascia and skin were sutured.

Administration of statins

In the PO administration group (PO group), rats ($n = 32$) were divided into four groups: (1) PO-control group, (2) PO-SV group, (3) PO-SVA group, and (4) PO-CVA group. In each group, (1) CMC solution only, (2) SV (10 mg kg^{-1} body weight/day) suspended in CMC solution, (3) SVA (10 mg kg^{-1} body weight/day) dissolved in CMC solution, and (4) CVA (0.3 mg kg^{-1} body weight/day) dissolved in CMC solution, were used. Each rat was administered the above reagents PO using feeding needles.

In the SC administration group (SC group), rats ($n = 32$) were also divided into four groups: (1) SC-control (CMC solution only) group, (2) SC-SV (SV 10 mg kg^{-1} body weight/day) group, (3) SC-SVA (SVA 10 mg kg^{-1} body weight/day) group, and (4) SC-CVA (CVA 0.3 mg kg^{-1} body weight/day) group. Each rat was administered the above reagents SC into the cervical region of the back.

The day of implantation was designated as day 0, and treatments were continued daily from day 1 to day 20. On day 21 after implantation, soft X-ray photographs (ED-125L; Shimadzu Co., Ltd, Kyoto, Japan) were taken to evaluate calcification at the implantation sites. All rats were then killed with an overdose of ether, and the BMP disks were removed.

Quantitative radiographic analysis

All disks were radiographed with a soft X-ray. The projected areas to the X-ray film (mm^2) and radiopaque areas with a trabecular pattern in the X-ray film (mm^2) were measured using NIH Image software (National Institutes of Health, Bethesda, MD, USA). Radiopaque areas measured the white areas in the disks that were scaled to a range of 0–128 (intermediate pixel value) in 256 columns.

Biochemical analysis

Six of eight disks from each group were weighed, frozen and stored at −80°C until assay. Disks were homogenized in 0.25 M sucrose with 0.3 M KCl in a homogenizer (Bio-Mixer, type SBM-1; Nissei Inc., Tokyo, Japan), and then centrifuged (12 000 ***g*** for 15 min at 4°C). Alkaline phosphatase (ALP) activity and tartrate-resistant acid phosphatase (TRAP) activity in the resultant aqueous supernatant were determined by the *p*-nitrophenyl phosphate method. The residues obtained by centrifugation of the sample homogenates were demineralized in 0.6 N HCl, and the calcium (Ca) content of the soluble fraction was determined by the *o*-cresolphthalein complexion method.

All values are presented as the mean ± standard deviation (s.d.). Statistical comparisons between groups were made using the Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

Histological examination

Two of eight disks from each group (one of seven disks in the SC-control group) were fixed with 10% neutral-buffered formaldehyde solution and demineralized with 10% ethylenediaminetetraacetic acid (pH 7.4) for 1 week at room temperature. After dehydration through graded ethanol solutions, the disks were embedded in paraffin. Approximately 6-μm-thick sections were cut and stained with hematoxylin and eosin for light microscopy.

Results

The weight of the rats increased in all groups, and the weight gain did not differ statistically between groups. One rat from the SC-control group died after 2 weeks of unknown causes. No illnesses occurred in the remaining rats.

Quantitative radiographic analysis

The radiopaque area and the density of the BMP disks obtained from the SC-SV group were both greater than those observed in the other SC and PO groups (Fig. 1). The projected area (Fig. 2a) and radiopaque area (Fig. 2b) from the X-ray film of the BMP disks obtained from the SC-SV group were significantly larger than those of the other SC groups, while no significant differences were noted among the PO groups. In particular, the radiopaque area of the SC-SV group was about 10 times larger than that of the other groups.

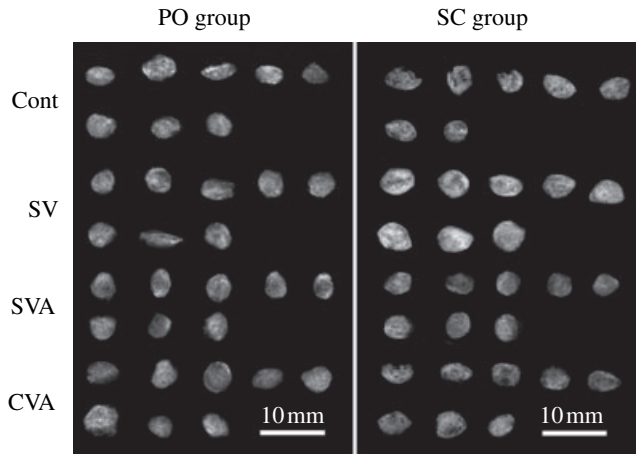

Figure 1 A soft X-ray photograph of all removed BMP disks on day 21 after implantation. PO, per oral administration; SC, subcutaneous administration; Cont, control group; SV, inactive lactone form of simvastatin group; SVA, active open hydroxy-acid form of simvastatin group; CVA, cerivastatin group. The radiopaque area and the density of the BMP disks obtained from the SV-SC group are larger and greater

Biochemical analysis

The ALP (Fig. 3a) and TRAP activities (Fig. 3b) in the disks obtained from the SC-SV group were significantly lower than those of the other SC groups, while no significant differences were demonstrated among the PO groups. The Ca content in the disks from the SC-SV group was significantly larger than that of the other SC groups. No significant differences were noted among the PO groups (Fig. 3c).

Histological findings

In all PO (data not shown) and SC groups, new bone formation was observed in the periphery of the BMP disk. In the SC-SV group, abundant trabecular bone with bone marrow was observed at the disk rim. Many osteoblasts were arranged on the bone surface and chondrocytes or cartilage remnants were not observed. The number of osteoclasts in the SC-SV group decreased compared with that of the other groups. A small amount of residual collagen still remained at the inside of the disk. In the other SC and the PO groups, a layer of mature trabecular bone was observed at the disk rim and residual implanted collagen was observed at the inside of the disks. No distinct morphological differences were noted among the other SC and PO groups (Fig. 4).

Discussion

This study is the first report to demonstrate the effects of statins on ectopic bone formation induced by rhBMP-2. The concentrations of SV and SVA (10 mg kg^{-1} body weight) were consistent with a previous report (Mundy *et al*, 1999). We also examined the influence of CVA,

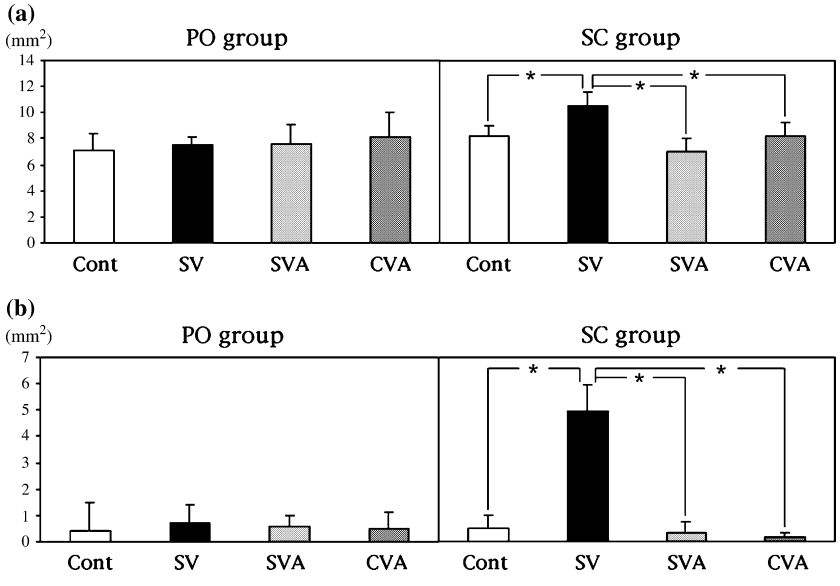

Figure 2 Quantitative radiographic analysis of BMP disks obtained from each group. (**a**) The projected area and (**b**) radiopaque area to the X-ray film. Data are expressed as the mean ± s.d. Differences between the groups are indicated by $*P < 0.05$ (Student's *t*-test). Both of the projected and radiopaque areas of the SV-SC group are significantly greater than those of the other SC groups, while no significant differences are noted among the PO groups

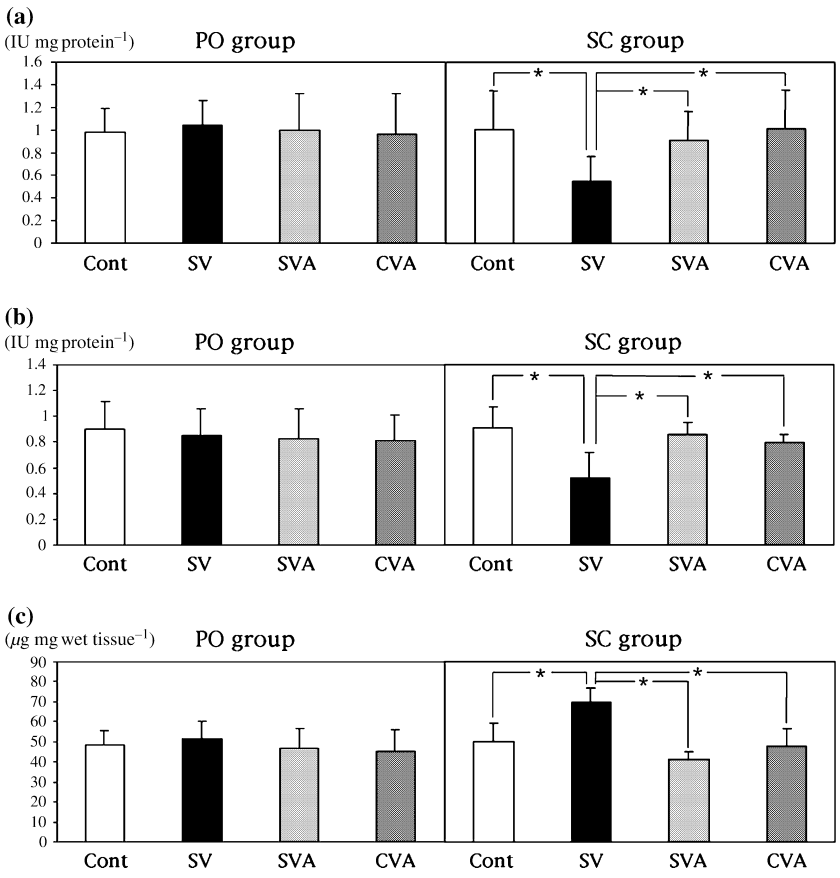

Figure 3 Biochemical analysis of BMP disks obtained from each group. (**a**) ALP activity; (**b**) TRAP activity; (**c**) Ca content. Data are expressed as the mean ± s.d. Difference between the group are expressed by $*P < 0.05$ (Student's *t*-test). In the SV-SC group, both of the ALP and TRAP activities are significantly lower and the Ca content is significantly larger than those of the other SC groups, while no significant differences are showed among the PO groups

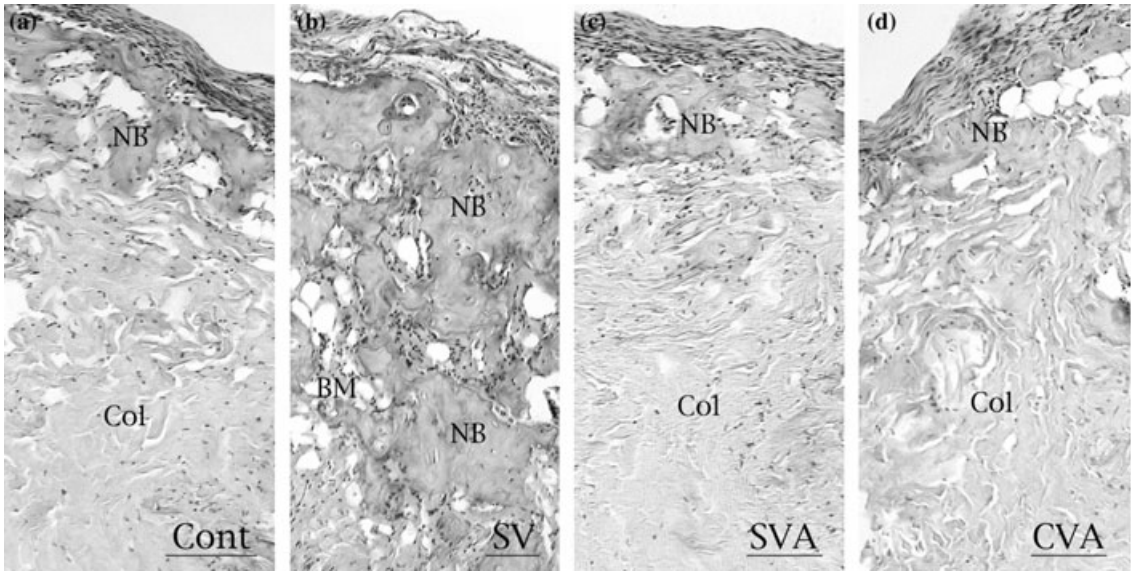

Figure 4 Light micrographs of the BMP disks obtained from the SC group on day 21 after implantation. (**a**) Control group; (**b**) SV group; (**c**) SVA group; (**d**) CVA group. NB, newly formed bone; BM, bone marrow; Col, residual implanted collagen. Hematoxylin and eosin stain, original magnification × 75. (**a**, **c**, **d**) A layer of mature trabecular bone has formed around the disk. Numerous collagenous remnants are seen inside the disk. (**b**) Abundant trabecular bone and bone marrow are present along with a small amount of residual collagen inside the disk. The number of osteoclasts decreased compared with that of the other groups. Neither chondrocytes nor cartilage remnants are found

which is almost three orders of magnitude more hydrophilic than SV (Garrett *et al*, 2001). The dose of CVA (0.3 mg kg^{-1} body weight) was established from a report that demonstrated that the HMG-CoA reductase inhibitory activity of CVA is about 30 times higher than that of SV (Dansette *et al*, 2000).

In the present study, we initially investigated the influence of PO administered statins (PO group). As SV is hydrolyzed into the active open hydroxy-acid form (SVA) after oral administration, the effects of SV, SVA and CVA were examined. However, these agents exhibited no significant stimulatory effects on the BMP-2 induction of bone. When statins are administered orally, a significant proportion of the dose accumulates in the liver and only a small proportion shifts to the systemic circulation. Accordingly, we attempted subcutaneous injection of statins (SC group) to avoid accumulation in the liver and to deliver the statins to the peripheral tissue. Ectopic bone formation was enhanced radiologically in the SC-SV group on day 21 after implantation, compared with those of the other SC groups. On biochemical analysis, the Ca content of the SC-SV group was higher, but ALP and TRAP activities of the SC-SV group were lower than those of the other SC groups. These data indicate that the degree of bone formation is superior to that of bone resorption under the situation of low bone turnover in the SC-SV group.

It has been argued whether the effect of oral statins is promotive on bone formation. Some clinical studies have found no significant effect of orally administered statins on bone (LaCroix *et al*, 2000; van Staa *et al*, 2000; Reid *et al*, 2001). *In vivo* studies by Maritz *et al* (2001) indicated that statins decrease BMD in the rat femur, when they were administered orally. One possible reason for these apparently disparate results is that orally administered statins easily accumulate in the liver and do not exert any effect on the bone. Demer (2001) stated that one of the reasons might be the influence of serum lipid levels on bone metabolism. However, SC administered statins do not likely accumulate in the liver, but circulate throughout the whole body, and would have no effects on blood cholesterol levels in the present study.

It is known that SV is more lipophilic and smaller in molecular weight than SVA or CVA (Garrett *et al*, 2001). These differences are reflected in the potential of each statin to nonselectively cross cellular membranes by passive diffusion (Hamelin and Turgeon, 1998). SV may easily penetrate blood vessels compared with SVA or CVA when they are injected SC. It is believed that statins can influence bone through the inhibition of HMG-CoA reductase. SV does not exhibit any HMG-CoA reductase inhibitory activity, but it is rapidly hydrolyzed to SVA by plasma esterase in rat (Vickers *et al*, 1990). While the plasma esterase activity in humans is lower than that in rats, the possibility remains that SV is transformed into SVA by intracellular enzymes such as osteoclastic esterases in the local bone microenvironment (Garrett *et al*, 2001). It has also been demonstrated that the SV-induced bone formation is similar to that of SVA or CVA in an organ culture study of murine neonatal calvaria (Garrett *et al*, 2001).

It has been demonstrated that bisphosphonates, which are known to be anti-bone resorptive drugs, act through inhibition of the mevalonate pathway, similar to the statins (Fisher *et al*, 1999). The inhibitory effect of bisphosphonates on bone turnover is greater than that of SV (Lips, 2002). However, to support the improvement of the quality and quantity of bone, it would appear that slow inhibition of osteoclasts or slow activation of osteoblasts is more desirable, as it maintains the coupling between bone formation and resorption. In addition, it has been demonstrated that bisphosphonates inhibit the maturation of BMP-2-induced ectopic bone, resulting in the retention of implanted collagen (Gong *et al*, 2003). In this study, there was only a small amount of residual implanted collagen and cartilage was replaced by trabecular bone in the SV-SC group. Therefore, SC administered SV may be more effective on ectopic bone formation by rhBMP-2 than bisphosphonates.

In conclusion, these results suggested that subcutaneous administration of SV stimulates ectopic osteoinduction by rhBMP-2 through the reduction of bone turnover. There is a possibility that SV may become a useful adjuvant for enhancing BMP-2-induced bone formation. It remains to be investigated whether SV really enhances the expression of endogenous BMP-2 in the BMP-2-induced bone in this experimental model. Elucidation of the influence of lipophilicity, dosage, duration of exposure and methods of administration of statins *in vivo* are necessary for future investigations.

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