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Original article

Effect of simvastatin on steroid-induced osteonecrosis evidenced by the serum lipid level and hepatic cytochrome P4503A in a rabbit model

Kentaro Iwakiri[1], Yutaka Oda[2], Yasunori Kaneshiro[1], Hiroyoshi Iwaki[1], Toshiaki Masada[1], Akio Kobayashi[1], Akira Asada[2], and Kunio Takaoka[1]

[1] Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
[2] Department of Anesthesiology and Intensive Care Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan

Abstract
Objective. This study was designed to investigate the efficacy of lipid-lowering agents in preventing steroid-induced osteonecrosis and the mechanism by which they do so in a rabbit model.
Methods. Female Japanese white rabbits were randomly allocated to receive probucol (group P), pravastatin (group PS), simvastatin (group SS), or saline (group C) for 6 weeks (n = 15 in groups P, PS, and SS; n = 30 in group C). Methylprednisolone (20 mg/kg) was injected at 3 weeks after starting treatment, and the femurs were histologically examined bilaterally 3 weeks after methylprednisolone injection. Midazolam clearance was measured before treatment and before methylprednisolone injection to determine hepatic cytochrome P4503A (CYP3A) levels.
Results. The incidence of osteonecrosis in the proximal metaphysis of the femurs in groups PS and SS was significantly lower than in group C ($P < 0.05$ and $P < 0.0001$, respectively), whereas it did not differ between groups P and C. It was significantly lower in group SS than in group PS ($P < 0.05$). Plasma concentrations of lipids (low-density lipoprotein, triglyceride, free fatty acid, and total cholesterol) in groups P, PS, and SS were significantly lower than in group C; and hepatic CYP3A levels were significantly higher in group SS than in groups P or PS after treatment ($P < 0.005$ for both).
Conclusions. Simvastatin and pravastatin significantly reduced the incidence of steroid-induced osteonecrosis in rabbits. Simvastatin was more effective in reducing the incidence of the disease, and increased CYP3A activity is a possible mechanism for this effect.

Introduction

Osteonecrosis of the femoral head (ONFH) is defined as the death of trabecular bone and bone marrow with resultant morbidity and disability of the hip joint. It is often associated with corticosteroid therapy.[1] Despite widespread use of corticosteroids for inflammatory diseases, the pathomechanism of the development of ONFH has not been identified and prevention of steroid-induced ONFH is currently difficult. Fat embolism in the intraosseous microcirculation due to steroid-induced hyperlipidemia has been suggested as a mechanism of steroid-induced ONFH,[2–6] and statins are effective in preventing ONFH by lowering intravascular lipid levels.[7,8] However, the variety of pharmacological effects of statins (e.g., improved blood circulation) suggest that other mechanisms in addition to lipid-lowering might contribute to preventing ONFH. Thus, comparing the efficacy of statins in preventing steroid–induced ONFH with those of other lipid-lowering agents is required to elucidate the mechanism.

Recent studies have shown that levels of hepatic cytochrome P4503A (CYP3A), a major drug metabolizing enzyme, are consistently low in patients with steroid-induced ONFH.[9] Because extrinsic glucocorticoids are metabolized and inactivated predominantly by hepatic CYP3A, individuals with low hepatic CYP3A activity are exposed to high levels of corticosteroids for prolonged periods of time, possibly leading to excessive effects or adverse events, including osteonecrosis. As hepatic CYP3A activity is inducible by various agents including statins,[10–13] increased CYP3A activity owing to statins may contribute to the prevention of osteonecrosis. In the present study, we examined the effects of individual statins on plasma lipid levels, hepatic CYP3A activity, and the incidence of steroid-induced osteonecrosis to elucidate the contribution of CYP3A to preventing osteonecrosis.

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Materials and methods

Experimental protocols

After approval from the Animal Care and Experiments Committee of Osaka City University, 75 adult female Japanese white rabbits (Japan SLC, Shizuoka, Japan) with ages ranging from 28 to 32 weeks were included in this study. Rabbits were housed in separate cages at the Animal Center in Osaka City University Medical School and were allowed free access to water and standard chalk food. Body weights were measured before experiments (range 3.1–4.2 kg) and every week during the experimental period.

The rabbits were randomly divided into four groups according to treatment prior to administration of methylprednisolone: (1) oral probucol (Daiichi-Sankyo, Tokyo, Japan) 200 mg/kg every other day (group P, $n = 15$); (2) intravenous pravastatin (Daiichi-Sankyo) 2 mg/kg daily (group PS, $n = 15$); (3) intravenous simvastatin (Toronto Research Chemicals, Toronto ON, Canada) 5 mg/kg daily (group SS, $n = 15$); (4) saline (group C, $n = 30$). Probucol, pravastatin, and simvastatin each have lipid-lowering effects. Probucol and pravastatin do not induce hepatic CYP3A, whereas simvastatin is a CYP3A inducer.[12] Each agent was administered for 6 weeks. The doses and durations of treatment of these agents were determined based on our preliminary study.

Methylprednisolone (Pfizer, Tokyo, Japan) (20 mg/kg) was injected into the right gluteus medius muscle 3 weeks after starting treatment, as previously described, to induce osteonecrosis.[14] All rabbits were euthanized by intravenous pentobarbital (1 mg/kg) 6 weeks after the start of treatment (3 weeks after injection of methylprednisolone). Bilateral femurs were harvested and were used to obtain mid-frontal and cross-sectional histological sections to detect osteonecrosis in the proximal metaphysis of the femurs, where the bone marrow necrosis is predominantly noted in this model.[14]

Measurement of serum lipid levels and midazolam clearance

Blood samples were collected from the auricular arteries while the rabbits were fasting. Samples were obtained in the early morning before treatment (day 0) and at 7, 14, 21, 24, 28, 31, 35, and 42 days after starting treatment. Low-density lipoprotein cholesterol (LDL), triglycerides (TGs), free fatty acids (FFAs), and total cholesterol (Tcho) levels were measured with an automated analyzer.

Hepatic CYP3A activity was evaluated by measuring midazolam clearance at the beginning of experiments and 3 weeks later using a method reported previously.[15] In brief, 9–11 rabbits were randomly selected from each group and were anesthetized by intramuscular ketamine and xylazine (10.0 and 1.2 mg/kg, respectively). After inserting a catheter into the auricular artery, midazolam (0.5 mg/kg) was administered intravenously via the auricular vein, and 1-ml blood samples were collected from the arterial catheter at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min after injection. The plasma concentration of midazolam was measured by one of the authors (Y.O.) who was unaware of the group allocation. Clearance of midazolam was calculated as dose divided by area under the plasma concentration–time curve with computer software (Sigma Plot version 10.0; Systat Software, San Jose, CA, USA).[15]

Preparation of histological sections and detection of osteonecrosis

The proximal femurs were fixed in 10% buffered formalin and decalcified in 14% EDTA:10% buffered formalin solution (pH 8). The decalcified femurs were dehydrated in gradient ethanol solutions and embedded in paraffin. The right side femurs were cut in the coronal plane and sections in the mid-coronal portion were used for histological identification of bone or bone marrow necrosis. The left femurs were sectioned vertical to the femoral axis, and sections of the femur were obtained just distal to the lesser trochanter. The sections were stained with hematoxylin and eosin (H&E) and were subjected to histological analysis to detect bone (marrow) necrosis.

The samples were examined in a blind fashion by two authors (K.I., Y.K.) independently. Bone and marrow necrosis was defined by the diffuse empty lacunae or pyknotic nuclei of osteocytes locating within the bone trabeculae or by eosinophilic bone marrow cell necrosis without normal morphology of marrow as reported previously (Fig. 1).[14] Based on the histological methods, the percent of animals with femurs with bone or marrow death lesions in the respective group were calculated.

Statistical analysis

Serum lipid levels were examined by one-way analysis of variance (ANOVA) with Scheffe's post hoc test. Midazolam clearance was analyzed by the unpaired *t*-test. The incidences of osteonecrosis in the proximal femurs were compared using the chi-squared test or Fisher's exact probability test. All analyses were performed with SAS statistical software (Version 9.1; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

The incidence of osteonecrosis in groups PS and SS was significantly lower than in group C ($P < 0.05$ and <0.0001, respectively), but did not differ between groups P and C (Fig. 2). It was significantly lower in group SS than in group PS ($P < 0.05$).

In all groups, levels of LDL, TGs, and Tcho began to increase 1 week after methylprednisolone injection. Levels of these lipids in groups P, PS, and SS were significantly lower than in group C at 2 weeks after methylprednisolone injection (group P: LDL $P = 0.0119$, TG $P = 0.0068$, Tcho $P = 0.0036$; group PS: LDL $P = 0.0019$, TG $P = 0.0152$, Tcho $P = 0.0028$; group SS: LDL $P = 0.0006$, TG $P = 0.0032$, Tcho $P = 0.0002$) (Fig. 3). Levels of LDL, TGs, FFAs, and Tcho were significantly lower in groups P, PS, and SS than in group C at 3 weeks after methylprednisolone injection ($P < 0.005$, <0.0344, and <0.005, respectively). There were no significant differences in the concentrations of LDL, TGs, FFAs, or Tcho among groups P, PSs and SS at any time point.

Before treatment, there were no differences in midazolam clearance among groups P, PS, and SS. After 3 weeks of treatment, this parameter was significantly elevated in group SS alone ($P < 0.005$) (not in group P or group PS). Midazolam clearance in group SS was significantly higher than those in groups P and PS ($P < 0.005$ for both) (Fig. 4).

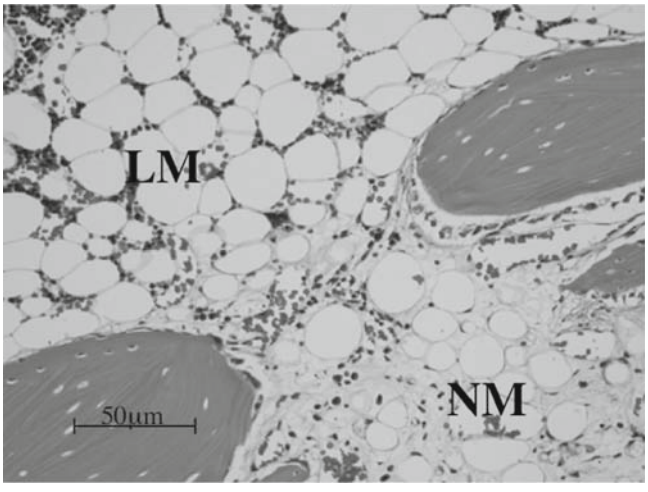

Fig. 1. Histological features of osteonecrosis in a rabbit model of steroid-induced osteonecrosis. Accumulation of bone marrow cell debris was observed in the necrotic bone marrow (*NM*). Slight appositional bone formation was noted between the necrotic bone marrow and living bone marrow (*LM*). H&E ×40

Discussion

In the present study, pravastatin and simvastatin significantly reduced the incidence of steroid-induced osteonecrosis in a rabbit model, but probucol did not. Because the plasma lipid (LDL, TG, FFA, Tcho) levels were comparable among animals receiving these three agents, it was clear that the reduced incidence of osteonecrosis by pravastatin and simvastatin was not due exclusively to their lipid-lowering activity. Hence, other mechanisms must have participated in this reduction.

We previously found that hepatic CYP3A levels were lower in patients with steroid-induced ONFH than in those with alcohol-induced ONFH and osteoarthritis, suggesting that low CYP3A might be a cause of ONFH.[9] As exogenous corticosteroid is predominantly metabolized by hepatic CYP3A,[16–18] low CYP3A activity would retard the metabolism of corticosteroids and expose the

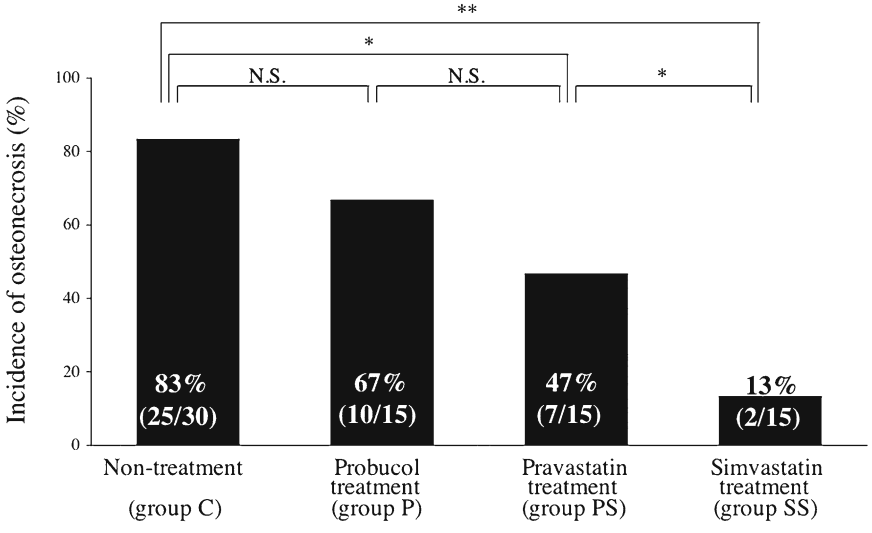

Fig. 2. Incidence of osteonecrosis in rabbits receiving probucol (*group P*), pravastatin (*group PS*), simvastatin (*group SS*), or saline (*group C*). The incidence of osteonecrosis in groups PS (47%) and SS (13%) was significantly lower than that in group C (83%) ($P < 0.05$ and <0.0001, respectively). It was significantly lower in group SS than in group PS ($P < 0.05$). *$P < 0.05$; **$P < 0.0001$

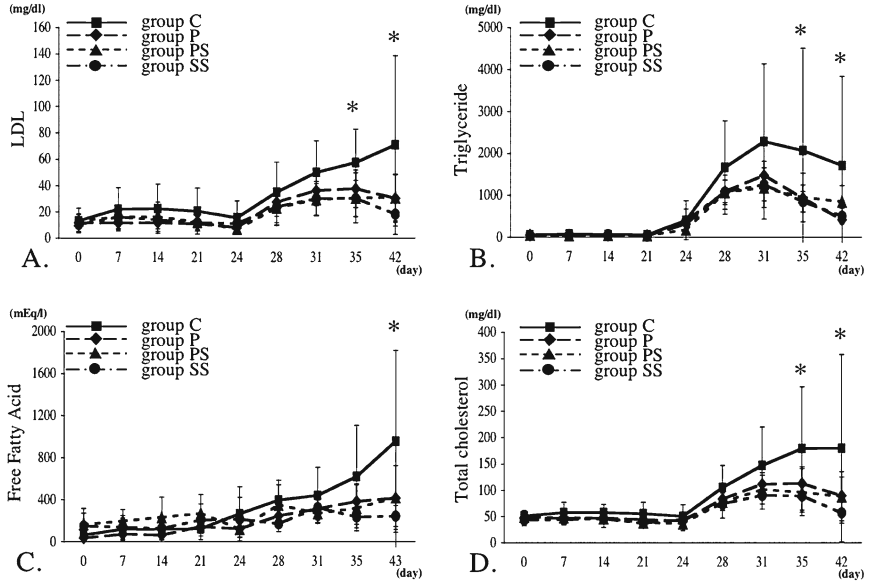

Fig. 3. Sequential changes in the levels of low-density lipoprotein (*LDL*) (**A**), triglyceride (TGs) (**B**), free fatty acids (FFAs) (**C**), and total cholesterol (Tcho) (**D**) in rabbits treated with probucol (*group P*), pravastatin (*group PS*), simvastatin (*group SS*), or saline (*group C*). There were no differences in the levels of LDL, TGs, FFAs, or Tcho among groups P, PS, and SS at any time point. $*P < 0.05$ compared with group C at the same time points

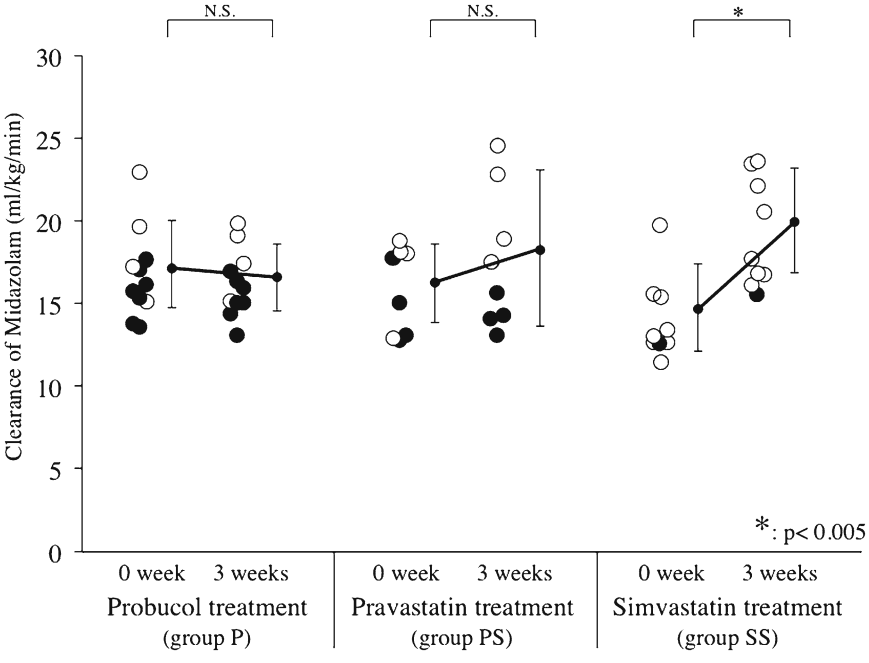

Fig. 4. Distribution profile of midazolam clearance before (*0 week*) and after 3 weeks (*3 weeks*) of treatment with probucol (group P), pravastatin (group PS), and simvastatin (group SS). Values are shown as individual values — *open circles*, osteonecrosis-negative; *filled circles*, osteonecrosis-positive) — and the mean ± SD (*small filled circles and whiskers*). There were no differences in midazolam clearance among groups P, PS, and SS before treatment. Midazolam clearance in group SS was significantly increased after treatment ($P<0.005$), whereas it was unchanged in groups P and PS

target tissues to high level of corticosteroids for longer durations of time, thereby inducing adverse reactions. In the present study, we compared the effects of two statins to elucidate the contribution of CYP3A activity in preventing osteonecrosis and found that only simvastatin increased the level of CYP3A and was more effective in reducing the incidence of steroid-induced osteonecrosis than pravastatin. These results suggest that inducing CYP3A activity as well as lowering lipid levels by simvastatin plays an important role in reducing the incidence of steroid-induced ONFH. This hypothesis is supported by our previous study, which showed that an increase in CYP3A activity reduced the onset of steroid-induced osteonecrosis in the same experimental model.[19]

Although our experimental data suggest that induction of CYP3A activity by simvastatin might reduce the risk for osteonecrosis, investigators should be careful when studying it clinically, as elevated CYP3A levels decrease the plasma concentration of corticosteroids

and reduce their effects. For patients with low CYP3A activity, doses of corticosteroids should be reduced to avoid ONFH rather than inducing CYP3A activity. Simple, reliable methods for measuring hepatic CYP3A activity are required for this purpose, and few methods meet these requirements.[20] Therefore, the development and validation of more simple and convenient methods that can be performed prior to corticosteroid therapy would be desirable to optimize the dose of corticosteroid for the individual patient. Such a method would be applicable to other drug therapy to avoid severe adverse events due to excessive drug action.

A major limitation of the present study is that osteonecrosis was predominantly induced in the proximal femoral metaphysis in the rabbit model, whereas it is commonly observed in the femoral head in ONFH patients. Furthermore, large doses of corticosteroids are required to induce osteonecrosis. However, the high incidence of bone and bone marrow necrosis induced in this model suggested that the model is suitable for investigating steroid-induced bone necrosis such as ONFH; and, in fact, it has been widely used for this purpose.[6,14,21–23] Secondly, we have examined only the effects of plasma lipids and CYP3A levels on the development of osteonecrosis; and the contribution of other possible mechanisms, such as impaired blood circulation in the bone, is not clear. Pharmacological effects specific to statins, such as improved endothelial barrier function and amelioration of oxidative stress, might have contributed to the reduced incidence of osteonecrosis.[24–27]

Conclusion

The incidence of steroid-induced osteonecrosis was significantly lower in rabbits receiving statins than in control rabbits. Furthermore, an increase in hepatic CYP3A activity by simvastatin significantly lowered the rate of osteonecrosis in rabbits compared with those treated by pravastatin, which is not a CYP3A inducer. It may be possible to reduce the risk of osteonecrosis by adjusting the dose of corticosteroids to the hepatic CYP3A activity in individual patients.

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