Effects of Cyclosporin A on Sex Hormone and Estrogen Receptor in Male Rat with Special Reference to Cyclosporin A-Induced Osteoporosis

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--- Abstract ---

The mechanisms of high turnover bone loss induced by Cyclosporin A (CsA) are not clearly understood. Deficiencies in sex hormones result in high turnover osteoporosis, and not only androgen but also estrogen plays an important role in maintaining bone mass in men. To study whether or not there are any changes in the levels of sex hormones, aromatization, and the expression of estrogen receptors in CsA-induced osteoporosis, we treated 39 rats with vehicle, low-dose CsA (5 mg/kg) and high dose CsA (15 mg/kg) for 28 days, and measured sex hormone levels by radioimmunoassay. Aromatase activities in ROS cells and 3T3-L1 cells were determined by measuring the conversion rate of \(^3\text{H}\)-androstenedione into \(^3\text{H}\)-estrone. ER and ER mRNA were measured by competitive RT-PCR in collected marrow cells and ROS cells. The levels of free testosterone in the serum in low-dose CsA-treated rats were unchanged, but the levels were significantly decreased in those treated with high-dose CsA as previously reported. The levels of total estradiol in the serum were significantly increased in the low-dose CsA-treated group (5 mg/kg) and were comparable to levels of the control group in the high-dose CsA-treated group (15 mg/kg). CsA increased the conversion of \(^3\text{H}\)-androstenedione to \(^3\text{H}\)-estrone in ROS cells, but not in 3T3-L1 cells. Meanwhile, CsA treatment did not change the rates of ER or ER mRNA expression in ROS cells or in collected bone marrow cells. In conclusion, CsA treatment decreased the level of free testosterone in the serum, but did not decrease the level of serum estradiol by enhancing aromatization. High-turnover osteoporosis induced by clinical dosage CsA treatment may not be caused by lowering the levels of circulating estrogen or by decreasing the expression of estrogen receptors.

--- Key Words: Cyclosporin A, osteoporosis, sex hormones, estrogen receptor, aromatase activity ---

INTRODUCTION

Cyclosporin A (CsA) is an immunosuppressive agent that alters the immune response by inhibiting the production and release of interleukin-2 and other cytokines, as well as T lymphocyte activation. There have been many clinical reports that CsA administered to organ transplant recipients gave rise to osteoporosis. CsA given in vivo has been associated with significant bone loss and increased bone remodeling. The changes of cytokines including TNF-α, IL-1, and TGF-β-1 have been suggested to be involved in CsA-induced osteoporosis. However, the mechanisms by which CsA induces high-turnover osteoporosis are not yet clear. Estrogen deficiency is one of the most important causes of osteoporosis in postmenopausal women. Significant bone loss in ERKO (estrogen receptor alpha knock-out) mice and in aromatase-deficient men strongly suggests that estrogen is also important in maintaining bone mass in men as well as in women. Even though the role of testosterone in the development of CsA-induced osteoporosis has been studied, little is known about the changes of estradiol levels and the expression of estrogen receptors after CsA treatment in vertebrate males. This study was designed to investigate the effects of CsA on sex hormones, especially serum estradiol, and ER and ER mRNA expression both in vivo and in vitro.

Our hypothesis was that a decrease of sex hormone levels or their receptors would be the main cause of high-turnover osteoporosis following CsA treatment. In contrast to our expectation, serum estradiol and
estrogen receptors did not decrease in this study.

MATERIALS AND METHODS

Materials

Thirty-nine 9-week-old male Sprague-Dawley rats, each weighing 290–330 g, were purchased from Korea Experimental Animal Centers (Seoul, Korea). All animals were housed under similar conditions at 18°C in a 12-h light/12-h dark cycle and maintained on a diet of Agway Prolab RMH 3000 (Agway, Syracuse, NY, U.S.A.), containing 0.75% calcium, 0.85% phosphorous, and vitamin D 1045 IU/kg and tap water ad libitum. CsA was provided by Sandoz Pharmaceuticals Corporation (Sandoz, Basle, Switzerland) in a solution containing 50 mg CsA/ml and 10% alcohol by volume in olive oil. The CsA solution was appropriately diluted in alcohol-olive oil to obtain a concentration of 50 mg/ml. The CsA dosage in vivo was based on the therapeutic dose range for CsA in rats for heart, kidney, and liver transplantation and graft-versus-host disease, as well as on previous studies demonstrating that CsA at a dose of 7.5–15 mg/kg could reliably be shown to cause high-turnover osteopenia. The concentrations of CsA on ROS cells and on 3T3-L1 cells were based on a previous report. Thirty-nine rats were randomly divided into 3 groups, labeled A, B, and C respectively. Group A rats (n=13) received vehicle (alcohol-olive oil), group B rats (n=15) received 5 mg/kg/day CsA, and group C rats (n=13) received 15 mg/kg/day CsA. CsA was administrated subcutaneously for 28 days.

Methods

To prepare total RNA from ROS 17/2.8 cells, ROS 17/2.8 cells were cultured in Ham’s F12 medium (GibcoBRL, Grand Island, NY, U.S.A.) containing 10% FBS and 1% antibiotics in a 100 mm plate, and almost confluent cells were used. For the thin-layer chromatography (TLC) assay, ROS cells and 3T3-L1 cells were cultured in 24-well tissue culture plates (Falcon plastics, Los Angeles, CA, U.S.A.). Culture medium for 3T3-L1 consisted of DMEM (GibcoBRL, Grand Island, NY, U.S.A.) with high glucose, 10% FBS and 1% antibiotics. On day 28, the rats were weighed and sacrificed by exsanguination. Blood was obtained via cardiac puncture. Blood then was centrifuged and the serum of each rat was stored at -20°C. To obtain bone marrow, both femurs of each rat were removed and the soft tissues were detached aseptically. Metaphysis from both ends was resected and bone marrow cells were collected by thrusting wooden sticks into bone. The bone marrow cells of each rat were collected in 1.5 ml microcentrifuge tubes and stored at -70°C. Bone marrow samples stored at -70°C were processed for total RNA isolation. Total RNA was extracted by using RNeasy Mini kit (Qiagen, Hilden, Germany). In case of total RNA of ROS cells and 3T3-L1 cells, total RNA was isolated by scraping the 100 mm dishes with a rubber policeman (Sigma, St. Louis, MO, U.S.A.) and extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). After quantification, RNA was diluted to 100 ng/μl in DEPC-treated H2O for RT-PCR. The standard ERα and ERβ template DNA were constructed as previously described. Competitive RT-PCR was done as explained below. The following reagents were added to a 500 μl microcentrifuge: 14.8 μl of D.W., 3 μl 10× buffer, 3 μl of 2 mM dNTP, 3 μl of 10 pmol primer, 1.5 μl of 100 mM DTT, 0.3 μl RNASin (Promega, Madison, WI, U.S.A.), 0.3 μl of AMV reverse transcriptase (Promega, Madison, WI, U.S.A.), 0.1 μl of Taq polymerase (Promega, Madison, WI, U.S.A.), 3 μl (3 μg) of extracted tissue RNA, and 1 μl of internal standard mRNA. The mixture was incubated at 42°C for 60 minutes and the reaction was stopped by boiling for 5 min at 95°C. Thirty-three cycles of PCR were done continuously. Each cycle consisted of 1 minute denaturation at 94°C, 1.5 minutes annealing at 62°C, and 2 minutes at 72°C for enzyme extension. Cycling was followed by a final extension at 72°C for 5 minutes. All samples were added to 1 μl 6× loading dye and the size was fractionated by electrophoresis in a 3.5% metaphor-agarose gel (FMC, Rockland, ME, U.S.A.). Visualization after ethidium bromide staining was performed and the amount of DNA band was analyzed. The RNA was serially diluted from 0 to 107 copy/μl (0, 105, 106, 107, 108 and 109 copy/μl), respectively. Products were visualized by running 10 μl of 100 μl total reaction volume on a 3.5% metaphor-agarose/1×TBE gel for 1.5 h at 100 V. The copy number of internal standard RNA was calculated by the following equation.
Copies/μl = OD_{260} \times 40 \times (ng/\mu l) \times 10^{-9} \times 6 \times 10^{23}/
\text{(standard bp} \times 660).\)

After titration of copy number of internal standard RNA, 1 μl of RNA (from 0 to 10^7 copies/μl) was added to each tube for RT-PCR reaction. Products were visualized by running 10 μl of 100 μl total reaction volume on a 3.5% metaphor-agarose/1×TBE gel for 1.5 h at 100 V. To confirm whether non-visualized products on 3.5% metaphor-agarose gel were not expressed at all, we repeated RT-PCR without any internal standard mRNA several times.

Free testosterone and total estradiol in the serum were measured by RIA using commercially available kits (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.). Aromatase activity was determined by measuring the conversion rates of [3H]-labelled androstenedione to [3H]-labelled estrone as previously described. Ninety-five percent confluent ROS cells and 3T3-L1 cells were treated with CsA or vehicle and were further incubated for 12 h and then treated with 25 nM [1,2,6,7^3H] androstenedione (3.52 TBq/mmol, Amersham, Little Chalfont, Buckinghamshire, UK). After another 12 h, the cell culture media were extracted with 4 volumes of methanol-ethyl acetate (1:4) and were again extracted 3 times with 4 volumes of ethyl acetate. The extracts were dried and dissolved in 50 μl of ethanol containing non-radioactive androstenedione and estrone for markers, and then applied to thin-layer plates (Whatman, Clifton, New Jersey, U.S.A.). Finally, they were chromatographed twice in chloroform-ethyl acetate (1:1, v/v). The marker steroids were located by dipping into the iodine chamber and the amounts of radioactivity on the spots of steroids were measured by β counter.

Statistical analysis

Statistical analyses were performed using the SPSS for Windows, release 7.5 (SPSS Inc., Chicago, Illinois, U.S.A.). All analyses were determined using the Students’ t-test. Throughout all analyses, a p value less than 0.05 was considered to be a significant difference.

RESULTS

Body weight and CsA concentrations in the serum

All groups gained weight over the 28-day experiment period. The high-dose CsA-treated group showed significantly lower body weight (354.17 ± 3.93 g) on day 28 compared to the vehicle-treated control group (371.67 ± 3.12 g) and low-dose CsA-treated group (375.39 ± 6.32, p < 0.05).

The serum CsA concentrations of the control group, low-dose CsA-treated group, and high-dose CsA-treated group were <25 ng/ml, 615 ± 46 ng/ml, and 1614 ± 49 ng/ml, respectively.

The effects of CsA on sex hormone in the serum

There was no significant difference in the free testosterone level in the serum between group A and group B, but the free testosterone level of group C was significantly decreased (Fig. 1). The free testosterone levels in the serum of each group were 15.19 ± 1.75 pg/ml, 12.10 ± 1.29 pg/ml, and 4.33 ± 1.39 pg/ml, respectively. There was a significant increase in estradiol level in the serum of group B compared to group A (Fig. 2). In contrast, the estradiol level in the serum of group C decreased compared to group B, and there was no significant difference between group A and group C. The mean estradiol level in the serum of each group was 12.54 ± 2.25 pg/ml, 23.95 ± 3.25 pg/ml, and 15.09 ± 2.87 pg/ml, respectively.

The effects of CsA on expressions of ER and ER in bone marrow and ROS cells

To determine the relative distribution of ER α and

![Graph](image_url)
ERβ mRNA in rat bone marrow, total RNA was extracted from bone marrow stored at -70°C. We quantified the expression of ER mRNA in rat bone marrow cells by competitive RT-PCR to detect both ERα and ERβ mRNA with internal standards. The amounts of expressed ERα mRNA were about 10^5 copy numbers in the control and CsA-treated groups (Fig. 3). In contrast, ERβ mRNA was hardly detected in any groups in competition with 10^5 copy numbers of internal standard ERβ mRNA (Fig. 3). RT-PCR without internal standard was done, but ERβ mRNA was hardly detected. Our results suggested that the estrogen receptors expressed in bone marrow cells were mostly ERα, and there was no significant change in the expression of ERα and ERβ mRNA before or after CsA treatment. ROS cells

**Fig. 2.** The levels of total estradiol in the serum after CsA treatment in rats. A significant increase in total estradiol in the serum was seen in the 5 mg/kg CsA-treated group compared to the control group and 15 mg/kg CsA-treated group (p<0.05).

**Fig. 3.** The expression rates of ERα and ERβ mRNA in the collected bone marrow cells after CsA treatment. No significant changes were observed in both ERα and ERβ mRNA expression. The sizes of the standard ERα and ERβ cDNA are 315 bp and 232 bp, respectively. The sizes of the target ERα and ERβ cDNA are 345 bp and 262 bp, respectively.

**Fig. 4.** The rate of ERα and ERβ mRNA expression after CsA treatment in ROS 17/2.8 cells, which was studied by competitive RT-PCR. A) The expression rate of ERα mRNA. The expression rate of ERα mRNA was -10^5 copies/μg total RNA. The upper band (345 bp) is the target ERα cDNA and the lower band is the ERα standard cDNA (315 bp). B) The expression rate of ERβ mRNA was -10^5 copies/μg total RNA. The upper band (262 bp) is the target ERβ cDNA and the lower band is the ERα standard cDNA (232 bp). M: 50 bp DNA ladder.
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**DISCUSSION**

In this study we have shown that the levels of estradiol in the serum does not decrease and that the expression rates of ERα and ERβ mRNAs remain unchanged in Csa-treated male rats, even though the levels of free testosterone in the serum decreased in a dose-dependent manner. Although Csa is an excellent immunosuppressive agent, bone loss in long-term users of this drug has been one of its adverse effects. 2-6 Csa produces a state of high-turnover osteopenia in rats and humans, which is dependent on both dose and duration. 9-10,12 A high turnover bone state is thought to be the result of a remodeling imbalance between osteoblastic and osteoclastic activity. The increase of 1,25 (OH)2D, TNFα, IL-1β, and other cytokines, and the decrease of testosterone have been suggested as possible explanations for Csa-induced osteoporosis, but the exact mechanisms are not yet clear. 7,8,11

The best-known example of high turnover bone loss is that caused by the deficiency of sex hormones. It is now clear that both estrogen and testosterone play important roles in maintaining bone mass in men and women. The level of free testosterone in the serum of postmenopausal women has a significant correlation with bone mineral density (BMD), and the combined treatment of estrogen and testosterone has resulted in a greater increase in BMD than estrogen treatment alone. 14,15,29,30 In addition, the development of osteoporosis in men with ER or aromatase gene point mutations has strongly suggested that estrogen also plays an important role in men. 30,31 The importance of sex hormone in Csa-induced osteoporosis has been previously studied, but these studies mainly focused on the decrease of testosterone in male vertebrates only. 17-22 Very few studies have been conducted on the change of estrogen level in Csa-induced osteoporosis in male rats. As expected, the level of free testosterone in the serum decreased significantly after high-dose Csa treatment, 17-22 but there was no significant difference after low-dose Csa treatment. The decrease in serum testosterone levels has already been reported to be due to the Csa-induced blocking of testosterone biosynthesis. 19 However, if Csa enhances the conversion of testosterone to estradiol, the enhanced conversion can also contribute to the decrease in serum testosterone. Here, we showed that there was some shift in the ratio of serum testosterone to estradiol after Csa treatment, even though it was premature to analyze the amount of contribution. The estradiol in the serum increased significantly in low-dose Csa therapy, whereas the
free testosterone in the serum remained unchanged. As well, total estradiol in the serum remained relatively constant in high-dose therapy compared to the control group, but the free testosterone in the serum decreased significantly. This was inconsistent with our expectation and the previous report that high-turnover bone loss is caused by a decrease in estrogen.\textsuperscript{28} The unexpected changes of estradiol in the serum following CsA treatment could be explained by the increased conversion of testosterone and estrone to estradiol. As it has been well established that androstenedione is the precursor of estrone and that P450 aromatase is the key enzyme in these conversions, we hypothesized that CsA might increase the aromatase activity in peripheral tissues. To test our hypothesis, we measured the conversion rates of estrone from androstenedione in the ROS and 3T3-L1 cell lines by using the TLC method. We found that the conversion rate increased almost 2.7 times at a high-dose CsA treatment (1 \(\mu\)g/ml) in ROS cells. However, it was not affected by CsA in the 3T3-L1 pre-adipocyte cell line, which has no aromatase activity.\textsuperscript{28} In support of our results, Gore-Lanton have already demonstrated that CsA increased aromatase activity in the granulosa cells of the ovary,\textsuperscript{24} but nothing was mentioned concerning peripheral tissues. The unchanged or even elevated estradiol levels in the serum following CsA treatment might have had some ameliorating effects on accelerated bone loss by a remarkable dropping of testosterone in the serum. Also, it may partially explain the results of previous studies where testosterone replacement therapy failed to prevent CsA-induced osteoporosis despite adequately maintained testosterone levels.\textsuperscript{17} As the effects of decreased testosterone levels might already be compensated for by the increase of estradiol in the serum, the addition of testosterone might therefore not make any significant difference. For a long time, estrogen receptor has been known to exist in only one form, ER\(\alpha\), but recently ER\(\beta\) has been cloned and is receiving much attention.\textsuperscript{32,33} However, the exact role of ER\(\beta\) is not yet clear, especially in tissues that have both receptors. High-turnover bone loss could develop even in the presence of adequate estrogen levels in the serum if the expression of ER\(\alpha\) is decreased significantly. To exclude the possibility that the expression of ERs are decreased in CsA-induced osteoporosis, we measured the expression of both ER\(\alpha\) and ER\(\beta\) by competitive RT-PCR in the bone marrow, one of the most important target organs of estrogen. Consequently, we detected no significant difference in ER expression in all groups. It was also true that there was no significant change in the levels of mRNA expression of both ER\(\alpha\) and ER\(\beta\) in CsA-treated ROS cells.

In conclusion, CsA treatment decreased the level of free testosterone in the serum, but did not decrease the level of estradiol by increasing aromatase activity, and the levels of ER\(\alpha\) and ER\(\beta\) mRNA expression were unchanged. Therefore, high-turnover osteoporosis induced by clinical-dosage CsA treatment may not be caused by the alternations in circulating estradiol or estrogen receptors, but rather by the changes of cytokines and local growth factors resulting from the direct and indirect effects of CsA on bone marrow cells. Our findings may help in developing a better understanding of the role of sex hormones and estrogen receptors in CsA-induced osteoporosis.

REFERENCES

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