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The Endothelin Receptor Antagonist, L-754,142 Does Not Prevent Cyclosporine A-Induced Osteopenia in Rats

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Abstract. Cyclosporine A (CsA) is a potent immunosuppressive agent widely used to prevent allograft rejection. *In vivo* administration of CsA is associated with the development of high-turnover osteopenia. Endothelin-1 (ET), a vasoconstrictive peptide, has been implicated in CsA-induced nephrotoxicty and hypertension. Recent evidence suggests that endothelin plays a pivotal role in bone metabolism. The present study was designed to investigate whether L-754,142 (ETRA), the combined endothelin A and B receptor antagonist, when given to rats, would favorably modify the bone loss caused by CsA. Fifty, 5-month-old male Sprague-Dawley rats were randomly divided into five groups of 10 rats each. The first group served as a basal control. The remaining four groups received, by daily gavage for 28 days, (1) a combined CsA and ETRA vehicle, (2) CsA, 10 mg/kg, (3) ETRA, 30 mg/kg, and (4) CsA, 10 mg/kg and ETRA, 30 mg/kg, respectively. Rats were weighed and venous blood was collected on days 0, 14, 28 for determination of BUN, creatinine, calcium, PTH, osteocalcin, and $1,25(OH)_2$ D. Tibiae, after double labeling, were removed following sacrifice for histomorphometry. Both CsA-treated rats and CsA/ETRA-treated rats demonstrated trabecular osteopenia with raised serum osteocalcin, and $1,25(OH)_2D$ levels when compared to control animals ($P < 0.05$). Rats given CsA alone developed renal impairment, as shown by an increased BUN. The combination group did not develop renal impairment. The results suggest that endothelin may contribute to the development of CsA-induced nephrotoxicity, which was prevented by ETRA, but does not seem to play a role in CsA-induced osteopenia.

Key words: Cyclosporine A — Endothelin — Receptor — Antagonist — Osteopenia.

In addition to the systemic hormones that affect bone, various local factors play an important role in the regulation of bone metabolism. The best studied of these are prostaglandins, cytokines, and growth factors [1, 2].

Endothelin (ET), which exists in three isoforms (1, 2, and 3) constitute a family of 21-amino acid vasoactive peptides widely distributed in many tissues including bone cells, elicits responses by biding to specific cell surface receptor subtypes A and B [3–5]. Biological effects to ET-1 on bone tissue include changes in alkaline phosphatase activity, regulation of osteocalcin and osteopontin mRNA expression, stimulation of collagen and noncollagenous protein synthesis, increased osteoblast proliferation [5a], increasing new bone formation in an osteoblastic tumor model [5b], inhibition of osteoclast motility, and stimulation of prostaglandin-dependent resorption [6]. ET-1 also enhances the interleukin-1-induced increase in IL-6, which is a potent stimulator of bone resorption [7]. Endothelins can also potentially affect calcium metabolism via inhibition of parathyroid hormone secretion [8]. Patients with active Paget's bone disease, a condition with accelerated bone turnover, demonstrated significantly higher ET-1 circulating levels than controls [9].

CsA, a fungal cyclic peptide, is an immunosuppressive agent that has become an integral component of treatment regimens used to prevent allograft rejection [10]. However, its use is associated with nephrotoxicity and hypertension [11]. Our laboratory has demonstrated that CsA causes accelerated bone loss in rats, which is both dose- and duration-dependent [12, 13]. The mechanism of CsA's effect on bone is complex and may involve the immune system. We have shown that T-lymphocytes play a critical role in the development of CsA-induced osteopenia [14], and CsA has been shown to increase mRNA expression of IL-1 and IL-6, cytokines known to be involved in bone resorption [15]. *In vivo* administration of CsA causes release of endothelin into the circulation from vascular endothelial cells and upregulates renal endothelin receptors [16–18].

Studies using endothelin receptor antagonists have been shown to inhibit cyclosporin-mediated endothelin side effects such as nephrotoxicity and hypertension [18, 19]. These observations suggest that endothelin could be one of the mediators of the CsA action on bone. Since CsA exerts its action on the kidney through endothelin, we hypothesized that the same peptide may be involved in CsA action on bone metabolism. We therefore examined the effect of oral administration of CsA in rats on bone to determine if

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this can be modified by the endothelin receptor antagonist, L-754,142-011w004.

Materials and Methods

Animals

Fifty, 5-month-old male Sprague Dawley rats weighing 500–650 g were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). All rats were housed under similar conditions and maintained on a diet of rat chow and tap water *ad libitum.*

Drugs

Endothelin receptor antagonist (L-754,142-001W004) ETRA in powder form was kindly provided by Dr. Peter Segal (Merck & Co, Rahway, NJ) and used as a 30 mg/ml solution in 0.5% methylcullulose for oral administration. Cyclosporine-A (100 mg/ml) was kindly provided by Novartis Pharmaceuticals (East Hanover, NJ) and diluted in an alcohol-olive oil vehicle to a working concentration of 10 mg/ml.

Experimental Protocol

Animals were randomly divided into 5 groups (1 basal and 4 experimental) of 10 animals each. The basal group received double labeling of demeclocycline 15 mg/kg b.wt on days −10 and −9, and calcein 10 mg/kg b.wt on days −2 and −1 by s.c. injection. These rats were sacrificed on day 0 to calculate the bone histomorphometry. The experimental groups were treated by daily oral gavage as follows: Group A: CsA vehicle and ETRA vehicle in equivalent volume (1 ml/kg b.wt); Group B: CsA (10 mg/kg b.wt) and ETRA vehicle in equivalent volume (1 ml/kg b.wt); Group C: ETRA (30 mg/kg b.wt) and CsA vehicle in equivalent volume (1 ml/kg b.wt); Group D: CsA (10 mg/kg b.wt) and ETRA (30 mg/kg b.wt).

All groups received double labeling of demeclocycline (Sigma) 15 mg/kg b.wt on days +18 and +19, and calcein (Sigma) 10 mg/kg b.wt on days +26 and +27 by s.c injection for determination of structural parameters of bone turnover.

The rats were weighted and bled on days 0, 14 (tail vein), and 28 (cardiac puncture) under ketamine 80 mg/kg b.wt i.m. (Fort Dodge Laboratories, Fort Dodge, IA). Blood was centrifuged and the sera stored at -80°C until assayed.

Assays

Serum osteocalcin was measured by radioimmunoassay (RIA) using a modification of a previously described procedure [20]. RIA reagents were obtained from Biomedical Technologies (Stoughton, MA). The sensitivity of the assay is 0.05–1.0 ng/ml, and intra- and interassay coefficients of variation are 7 and 8%, respectively.

Serum parathyroid hormone (PTH) was measured by a two-site immunoradiometric (IRMA) using a commercially available kit (Nichols Institute Diagnostics, San Juan Capistrano, CA), which utilizes two different goat antibodies to the N-terminal region (1–34) of rat PTH; one antibody immobilized onto plastic beads to capture the PTH molecules, and the other radiolabeled for detection. The immobilized antibody binds both PTH (1-84) and N-terminal PTH (1-34). Coefficients of variation are 4.0–4.3% for intraassay precision and 4.3–4.7% for interassay precision. Our laboratory has previously confirmed the validity of this assay in the rat [21].

Serum 1,25-dihydroxyvitamin D (1,25$(OH)_2$ D) was assayed using a commercially available kit (DiaSorin, Stillwater, MN, USA). Vitamin D metabolites were extracted and purified using C_{18}OH and silica cartridges. The assay was then performed using a competitive RIA procedure which is based on a polyclonal antibody that is specific for both 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$. Coefficients of variation are 9.7–14.1% for intraassay precision and 14.7–26.0% for interassay precision.

Histological techniques

The following histomorphometric parameters of proximal tibial metaphysis were determined as previously described [22, 23]:

1. Percent trabecular area (%Tb.Ar), indicating the percentage of total cancellous bone within the total measured area
2. Trabecular thickness (Tb.Th), indicating the thickness of trabeculae in cancellous bone
3. Trabecular number (Tb.N), indicating the number of trabeculae within the area of cancellous bone measured
4. Trabecular separation (Tb.Sp), indicating the separation of trabeculae within the area of cancellous bone measured.

These measurements will indicate amount of bone formation, amount of bone resorption, and the net effect of these two processes.

Statistical Analysis

The data were analyzed by one-way ANOVA (with Bonferroni correction) at each time point (day 0, 14, and 28). A P value less than 0.05 was considered statistically significant. Bone histomorphometric data were analyzed by comparing the basal group with the experimental control group using independent samples t-tests to ensure that the vehicle used did not modify baseline histomorphomeric indices. Drug-treated groups were compared with the control (vehicle) group using one-way ANOVA (with Bonferroni correction) for each variable.

Results

Table 1 is a summary of serum BUN, creatinine, and calcium levels in all experimental groups. CsA-treated rats had significantly higher ($P < 0.05$) BUN levels on days 14 and 28 compared with that of control animals, but levels in the CsA/ETRA-treated group remained within normal range. There were no significant differences in serum creatinine and total calcium levels between the groups. Body weights were statistically indistinguishable.

Table 2 shows the histomorphometric data obtained on day 28 for tibiae samples from all groups. The results show that CsA, as expected, significantly decreased trabecular volume and thickness and increased trabecular separation, compared with controls, indicating bone loss which was not influenced by combining CsA with ETRA. Intergroup comparison showed that trabecular volume and separation were significantly lower in the combined CsA/ETRA group than in CsA alone ($P < 0.05$). In fact, there appears to be a worsening (increasing) effect of the combined treatment. Although trabecular number was numerically less in the CsA-treated and combination group, this difference did not reach statistical significance. ETRA by itself had no effect on these parameters of bone remodeling. Figure 1 shows the effects of CsA, alone or in combination with ETRA, on serum osteocalcin levels. CsA increased serum osteocalcin, as expected, but this increase was not affected by endothelin receptor blockade.

Serum PTH levels did not change significantly over the

Table 1. Effect of oral administration of CsA, alone or in combination with ETRA, on body weight, serum BUN, creatinine, and total calcium levels in the rat

Parameter	Day	Vehicle	CsA (10 mg/kg)	ETRA (30 mg/kg)	ETRA/CsA
Weight (g)	0	566 ± 24	641 ± 24	613 ± 24	587 ± 17
	14	595 ± 21	649 ± 32	603 ± 27	606 ± 21
	28	593 ± 21	648 ± 29	613 ± 27	536 ± 64
BUN (mg/dl)	0	17.9 ± 1.1	17.3 ± 0.8	15.3 ± 0.6	16.2 ± 1.1
	14	17.1 ± 1.2	21.8 ± 1.6[a]	14.1 ± 0.7	16.7 ± 1.6
	28	19.7 ± 1.2	25.3 ± 2.4[a]	18.3 ± 0.7	19.3 ± 1.5
Creatinine (mg/dl)	0	0.31 ± 0.02	0.33 ± 0.02	0.29 ± 0.03	0.30 ± 0.04
	14	0.30 ± 0.02	0.35 ± 0.02	0.22 ± 0.02	0.32 ± 0.05
	28	0.42 ± 0.03	0.42 ± 0.02	0.42 ± 0.02	0.43 ± 0.03
Total calcium (mg/dl)	0	8.33 ± 0.36	7.98 ± 0.29	7.52 ± 0.35	7.28 ± 0.37
	14	8.52 ± 0.58	7.92 ± 0.35	6.88 ± 0.43	7.44 ± 0.44
	28	8.76 ± 0.36	8.61 ± 0.13	9.04 ± 0.39	8.60 ± 0.42

Values reported are means (±SEM)
[a] Significantly different from other groups ($P < 0.05$)

Table 2. Effect of oral administration of CsA, alone or in combination with ETRA, on structural parameters of bone histomorphometry

Parameter	Basal	Vehicle	CsA (10 mg/kg)	ETRA (30 mg/kg)	CsA/ETRA
Trabecular vol. (%)	23.6 ± 1.5	20.1 ± 1.3	15.8 ± 1.1[a]	20.8 ± 1.4	12.7 ± 1.7[ab]
Trabecular Th. (μm)	75.5 ± 3.1	74.2 ± 4.0	60.9 ± 3.7[a]	65.9 ± 4.4	62.1 ± 6.2[a]
Trabecular no. (/mm)	3.1 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	3.2 ± 0.2	2.1 ± 0.3
Trabecular sep. (μm)	325 ± 13	365 ± 17	397 ± 17	311 ± 18	517 ± 59[ab]

Values reported are means (±SEM)
[a] Significantly different from vehicle-treated and ETRA-treated groups ($P < 0.05$)
[b] Significantly different from CsA alone group ($P < 0.05$)

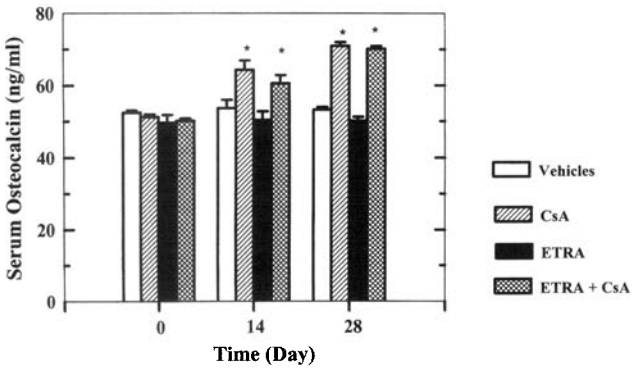

Fig. 1. Effects of oral administration of CsA (10 mg/kg b.wt.), alone or in combination with ETRA (30 mg/kg b.wt) on serum osteocalcin (BGP) levels in rats. (* Significantly different from vehicle-treated and ETRA-treated groups; $P < 0.05$, ANOVA).

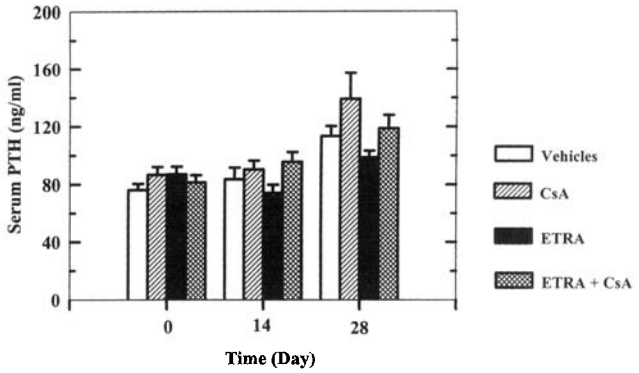

Fig. 2. Effects of oral administration of CsA (10 mg/kg b.wt.), alone or in combination with ETRA (30 mg/kg b.wt) on serum PTH levels in rats.

study period (Fig. 2). Both the CsA-treated group and CsA/ETRA-treated group had significantly elevated $1,25(OH)_2D$ levels on day 28 ($P < 0.05$) compared with the control group (Fig. 3).

Discussion

Based on bone histomorphometry, CsA alone and the combination group showed increased bone turnover with a decreased bone mass, which was worse in the combination group. ETRA was unable to prevent bone loss induced by CsA, and may have aggravated it, although this is difficult to prove, as ETRA by itself had no effect. Tsukahara et al. [24] have shown that administration of wFR139317 (50 mg/kg b.wt), a specific ET (A) receptor antagonist, caused a significant decrease in bone mass in the lumbar spine of

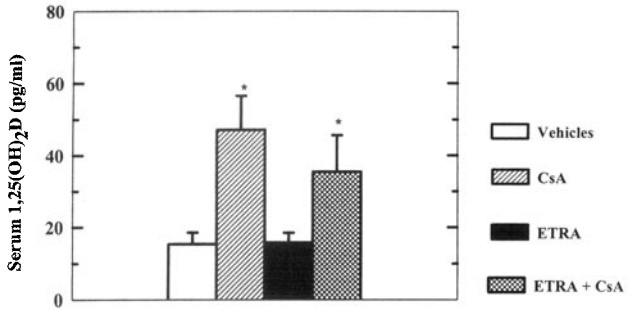

Fig. 3. Effects of oral administration of CsA (10 mg/kg b.wt.) alone or in combination with ETRA (30 mg/kg b.wt) for 28 days on serum 1,25-dihydroxyvitanin D levels in rats. (* Significantly different from vehicle-treated and ETRA-treated groups; $P < 0.05$, ANOVA).

growing rats and this was associated with a decrease in the serum osteocalcin [24]. In that study, both the endothelin receptor antagonist (specific for $ET_{(A)}$receptor) and the dose used were different from that used in our study. Another confounding issue is that the age of the animals was also different and thus comparison is not really valid. In contrast to *in vitro* [5a] and *in vivo* [5b] studies where ET stimulated osteoblast proliferation and new bone formation, and administration of ETRA decreased these responses, we found no effect of ETRA alone in our rat studies. This may well be related to a dose and frequency schedule.

Osteocalcin is secreted *in vivo* by differentiated osteoblasts and is a useful marker for skeletal growth and overall bone turnover [25]. *In vitro* findings suggest that induction of osteocalcin may be mediated by endothelin A receptors, and these receptors are also down-regulated at the mRNA level by $1,25(OH)_2$ vitamin D_3 [26]. In our study, serum osteocalcin was increased in both the CsA-treated and CsA/ETRA-treated groups. That the increased osteocalcin is not due to decreased renal clearance with CsA nephrotoxicty was demonstrated by the fact that the combination group also showed an elevated level without renal impairment. *In vivo* administration of ETRA, alone or in combination with CsA, did not change osteocalcin levels, suggesting that raised osteocalcin level is not related to endothelin action on osteoblasts. A possible explanation for this is that some other local factors might play a role in modifying endothelin receptor blockade *in vivo*.

As described clinically [27] and experimentally [17], the CsA-treated group showed renal impairment, with an increased BUN compared with the combination group. This finding was expected as the endothelin-receptor antagonist has been shown to be protective *in vivo* against acute CsA toxicity [18], and is evidence that the ETRA was active. Serum creatinine did not change, therefore the renal impairment was not severe.

Previous studies have documented the presence of both ET A and B receptors in human parathyroid tissue through which ETs may modulate PTH secretion in an autocrine and/or paracrine fashion [28]. Although in the present study serum PTH was elevated at the end of the study in all groups (day 28), there were no significant differences between groups. There was no alteration of total calcium levels, suggesting that renal osteodsytrophy could not account for the bone changes that occurred with CsA.

Serum $1,25(OH)_2D$ was elevated on day 28 in both CsA-treated and the CsA/ETRA-treated groups, which is consistent with our previous report that CsA increases 1α-hydroxylase activity and therefore produces significant elevations in $1,25(OH)_2D$ levels [29]. Again, endothelin did not appear to play a role in the CsA-induced increases in serum $125(OH)_2D$ levels.

The study indicates that endothelin may not be a factor in mediating the CsA-induced bone loss. Although it relied upon indirect evidence using ETRA to block the action of endothelin, the fact that it prevented an increase in CsA-induced renal impairment indicates an *in vivo* effect, which did not translate to a bone action. An alternative explanation for why ETRA protected the kidney against CsA toxicity but not bone may be that the kidney cells are more sensitive than bone cells and larger doses may be required to observe an effect. It is also interesting that the increase in osteocalcin and $1,25(OH)_2D$ was not influenced by ETRA, suggesting that these markers for bone metabolism are not ET-mediated.

In conclusion, endothelin may be involved in CsA nephrotoxicity and normal bone metabolism but it has no role in CsA-induced action on bone.

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