Cadmium accelerates bone loss in ovariectomized mice and fetal rat limb bones in culture
(postmenopausal osteoporosis/smoking/Itai-Itai disease/parathyroid hormone)

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ABSTRACT Loss of bone mineral after ovariectomy was studied in mice exposed to dietary cadmium at 0.25, 5, or 50 ppm. Results show that dietary cadmium at 50 ppm increased bone mineral loss to a significantly greater extent in ovariectomized mice than in sham-operated controls. These results were obtained from two studies, one in which skeletal calcium content was determined 6 months after ovariectomy and a second in which 45Ca release from 45Ca-prelabeled bones was measured immediately after the start of dietary cadmium exposure. Furthermore, experiments with 45Ca-prelabeled fetal rat limb bones in culture demonstrated that Ca at 10 nM in the medium, a concentration estimated to be in the plasma of mice exposed to 50 ppm dietary Cd, strikingly increased bone resorption, from 27 ± 2% (mean ± SEM) 45Ca release in cultures with no added cadmium to 68 ± 6% release in cultures containing cadmium (n = 4). These in vitro results indicate that cadmium may enhance bone mineral loss by a direct action on bone. Results of the in vivo studies are consistent with a significant role of cadmium in the etiology of Itai-Itai disease among postmenopausal women in Japan and may in part explain the increased risk of postmenopausal osteoporosis among women who smoke.

Osteoporosis affects 15–20 million people in the United States and is a major cause of bone fractures in older persons, particularly postmenopausal women (1). One striking form of postmenopausal bone disease has occurred among women in Toyama prefecture, Japan, in the form of Itai-Itai (Ouch-Ouch) disease, which is characterized by severe osteoporosis/osteomalacia and renal tubular dysfunction (2–5). Elevated levels of cadmium were present in both the diet and drinking water of women with Itai-Itai disease. The Japanese Ministry of Health in May 1968 declared cadmium to be one of the causative factors in the etiology of this bone disease on the basis of epidemiologic data relating disease incidence to cadmium exposure and the finding of high cadmium levels in tissues taken at autopsy from women with the disease (4, 6).

The studies reported here were designed on the basis of the unusual finding that 95% of the cases of Itai-Itai disease occurred in postmenopausal women, not in younger women, men, or children (4). Although many studies of cadmium-induced bone loss had been conducted in male animals or in females with intact ovaries, none had addressed the question of whether the combination of cadmium exposure and ovariectomy (to simulate conditions of postmenopausal ovarian hormone depletion) might accelerate the loss of bone calcium that normally occurs after removal of the ovaries. Such results might explain why Itai-Itai disease did not appear until after menopause.

Results of our studies show that loss of bone mineral after ovariectomy in female mice was strikingly increased by dietary cadmium exposure. In addition, when cadmium was added at low concentrations (10 nM) to bone cultures, bone resorption was stimulated to an extent similar to that observed after treatment of the cultures with parathyroid hormone, the endogenous bone resorbing agent. The in vivo results provide an animal model for research into one mechanism of increased postmenopausal bone loss. The in vitro results indicate that cadmium may accelerate bone loss by a direct action on bone. Because cadmium concentrations in the blood of smokers are comparable to those in both our in vivo and in vitro studies (7), our results further suggest that cadmium in cigarette smoke in combination with smoking-induced decreases in estrogen levels (8, 9) may be one cause of the decreased bone mineral content, increased incidence of fractures, and earlier tooth loss observed in women who smoke (10–14).

MATERIALS AND METHODS

Cadmium-Induced Bone Loss in Ovariectomized Mice: Stable Calcium Study. Virgin female CF1 mice (Charles River Breeding Laboratories) were fed purified diets containing cadmium chloride at either 0.25, 5.0, or 50 ppm Cd. The purified diet was based on the American Institute of Nutrition AIN-76A diet. Calcium (0.52%), phosphorus (0.56%), and vitamin D (1 international unit/g) levels were adequate but not excessive. Mice exposed to cadmium at 0.25 ppm constituted the environmental exposure group; cadmium at this concentration is present in the stock Wayne Lab Blox diet that the mice consumed prior to the start of the study. Mice were introduced to their experimental diets at 70 days of age. After 12 months of exposure, virgin females were ovariectomized (OV) or sham-operated (SO). Surgery was carried out under intravenous pentobarbital anesthesia combined with inhalation exposure to methanethiol. A 1-cm midline abdominal incision allowed for exposure and surgical removal of the ovaries of the OV mice. SO mice were anesthetized, and ovaries were exposed but not removed.

After surgery, OV and SO females were maintained on their respective diets for an additional 6 months before sacrifice. At sacrifice, lumbar vertebrae (L1–L5) and femurs were removed, and their dry weight, ash weight, and calcium content were determined as described previously (15). Thoracic vertebrae (T12–T14) were removed, cleaned of extraneous soft tissue, fixed in formalin, and defatted by four successive changes of anhydrous diethyl ether/methanol, 1: 1 (vol/vol). Microradiographs were prepared by embedding the vertebrae in methylmethacrylate, cutting 100-μm-thick cross sections at the midpoint of the vertebrae, and exposing the

Abbreviations: OV, ovariectomized; SO, sham-operated; PTH, parathyroid hormone.

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sections to x-ray plates, and exposing them to low-energy x-rays (12 keV; 1 eV = 1.602 x 10^{-19} J).

**Cadmium-Induced Bone Loss in Ovariectomized Mice: 45Ca Study.** The bone calcium of 44-day-old virgin female CF1 mice was labeled with 45Ca. Over a 1-week period, each mouse received three intraperitoneal injections, each containing 9.0 x 10^{6} Bq (6.9 µCi/ml, New England Nuclear) in 2 ml of 0.15 M NaCl. Beginning 1 month after the first 45Ca injection, all mice were housed individually in stainless steel metabolism cages and given the purified AIN-76A diet with no added cadmium. One week later, urine and feces were collected from each animal for two nearly consecutive 96-hr periods in order to obtain measurements of 45Ca excretion, which in turn provided a measure of 45Ca release from bone (16). Excreta were collected on plastic-backed absorbent paper placed under the wire-grid cage bottoms. Mice were weighed, and food consumption was measured for each mouse during each period of excreta collection.

After the 96-hr baseline collections, surgery was performed to either remove (OV mice) or expose (SO mice) the ovaries. Starting immediately after surgery, urine and feces were again collected from each mouse for three 96-hr periods. After these three collections, one-half of the OV mice and one-half of the SO mice were introduced to the AIN-76A diet containing 50 ppm Cd. The remaining mice stayed on the AIN-76A diet without cadmium. After the switch to the cadmium-containing diet, three consecutive 96-hr collections were made to determine if there was any immediately discernible effect of cadmium exposure on 45Ca excretion.

At the time of excreta collection, the feces from each mouse were removed from the paper and placed in a beaker: the urine spots were cut from the absorbent paper. Fecal samples were ashed in a muffle furnace for 48 hr at 450°C, digested in concentrated HNO_{3}/hydrogen peroxide, brought to incipient dryness, dissolved in 3 M HCl, and diluted 1:2 with water. Aliquots of the fecal digest were removed, and their 45Ca contents were determined with a Beckman model 3150T liquid scintillation counter by measuring the ß-decay at ~70% efficiency. Additional aliquots were digested into 0.1 M HCl/0.1% lanthanum chloride and analyzed for calcium by flame atomic absorption spectrophotometry. The urine samples from each mouse were placed in a beaker, and the calcium in the urine was leached from the papers for 24 hr into 0.1 M HCl/0.1% lanthanum chloride. Aliquots of the urine extracts were analyzed for 45Ca content by liquid scintillation spectrometry as described for the fecal samples. Additional aliquots of urine were digested into 0.1 M HCl/1% lanthanum chloride and analyzed for calcium by flame atomic absorption spectrometry. The higher lanthanum chloride concentration (1% vs. 0.1%) was required because of the high phosphate-to-calcium ratio in urine. The 45Ca contents of all samples were corrected for decay to the first day of 45Ca injection.

**Fetal Rat Limb Bones in Culture.** Timed-pregnant Sprague-Dawley rat dams (Holtzman, Madison, WI) were administered 2.6 x 10^{6} Bq (200 µCi) of 45CaCl_{2} by intraperitoneal injection on day 18 of gestation. On day 19 of gestation, forelimb bones were obtained from the fetal rats. Midshfts were dissected and incubated in modified BGJ medium containing 0.1% bovine serum albumin (pH 7.3) under 5% CO_{2}/95% air, as previously described (17). Cadmium (0.01 or 1 µM CdCl_{2} and/or parathyroid hormone (PTH; 1 or 10 nM PTH, Bachem, Torrance, CA) were added at the start of the incubation period. After 60 hr of incubation, each tissue was extracted in 0.5 ml of 0.5 M HCl. 45Ca in culture medium and bone extracts was measured by liquid scintillation spectrometry.

**Statistical Analyses.** Statistical differences were determined by analysis of variance followed by the least-significant-difference test.

**RESULTS**

Effect of Cadmium on Loss of Bone Mineral in OV Mice. In the stable calcium study, mice at all levels of dietary cadmium grew at similar rates during the first 170 days of exposure (70-240 days of age). Body weights then gradually increased more slowly in the mice receiving 50 ppm Cd such that, at sacrifice, these mice were 20-25% lighter than the mice receiving the environmental cadmium level (0.25 ppm). In contrast, femur lengths at sacrifice were nearly the same for mice in all groups, with no statistically significant decrease due to cadmium exposure (Fig. 1; compare the 50 ppm Cd/SO group with the 0.25 ppm Cd/SO group and the 50 ppm Cd/OV group with the 0.25 ppm Cd/OV group), indicating that treatment-related differences in body weight did not reflect differences in the size of the skeleton. During the 6-month period from surgery to sacrifice, during which time ovariectomy-induced decreases in bone mineral content/concentration occurred, changes in mean body weight were small in all groups: a 5-10% increase in OV mice and a 0-5% decrease in SO mice for all cadmium exposure levels. Therefore, ovariectomy-induced decreases in bone calcium (Fig. 2) could not be attributed to ovariectomy-induced decreases either in body weight or skeletal growth (Fig. 1).

The mean calcium content of femurs from OV mice at 0.25 ppm dietary Cd was 14% lower than that of femurs from SO mice (Fig. 2 Upper, 0.25 ppm Cd/OV group vs. 0.25 ppm Cd/SO group), in agreement with the effects of ovariectomy on bone reported by others (18, 19). In our experiment, however, this decrease was not statistically significant by the analysis of variance and the least-significant-difference test and did not occur in the mice exposed to cadmium at 5 ppm (Fig. 2). In contrast, exposure to 50 ppm dietary Cd caused a significant increase in the loss of bone calcium after ovariectomy such that the calcium contents and calcium-to-dry-weight ratios (data not shown) of both femurs and lumbar vertebrae of the OV mice at 50 ppm dietary Cd were strikingly lower than those of nearly all other groups (Fig. 2). For example, the femurs of OV mice receiving 50 ppm dietary Cd contained a mean of only 9.6 mg of Ca, whereas femurs from the other groups contained 14-17 mg of Ca (Fig. 2 Upper). Furthermore, microradiographs of the cross sections of thoracic vertebrae showed that the calcium contents of the bones from OV mice exposed to 50 ppm dietary Cd were clearly lower than those of the other groups (Fig. 3). Results of this study demonstrate that dietary Cd at 50 ppm decreased the calcium content of mouse bones after ovariectomy. The mice in this study were quite old (435 days old at ovariectomy; 617 days old at sacrifice) and had been exposed
to dietary cadmium for one year prior to removal of the ovaries. Cadmium has been shown to cause renal damage after prolonged exposure to high levels of dietary cadmium (2, 5).

Some investigators propose that cadmium-induced loss of bone mineral occurs as a secondary response to cadmium-induced renal tubular degeneration (20–22). Consequently, the 45Ca study described in Materials and Methods was conducted to determine whether cadmium exposure would cause an increased release of 45Ca from the skeletons of OV mice immediately after the start of cadmium exposure and in the absence of the 1-year preexposure period present in our first study.

As shown in Fig. 4, during the first two periods of excreta collection, which took place prior to both surgery and cadmium exposure, fecal 45Ca excretion (a measure of 45Ca release from bone) decreased with time in mice of all groups; no consistent differences between groups were observed.

During the third collection period, which was the first period after ovariectomy, no effect of ovariectomy on fecal 45Ca excretion had yet occurred. However, during the fourth and fifth rounds of excreta collection (the second and third rounds after ovariectomy), a clear increase in fecal 45Ca excretion occurred in the OV mice compared to the SO controls (Fig. 4). During the fifth round, OV mice excreted 59 ± 7% (mean ± SEM, n = 10) more 45Ca into feces than did the SO mice.

After the fifth round of collection, one-half of the OV and one-half of the SO mice were switched to diets containing 50 ppm Cd. As shown in Fig. 4, cadmium exposure caused an increase in fecal 45Ca excretion in both the OV and SO mice. In addition, the cadmium-induced increase was clearly greater in OV than SO mice: in the OV mice, fecal 45Ca excretion during the first collection after the start of cadmium exposure was 26 ± 4% (n = 10) greater than during the collection just before cadmium exposure; by comparison, in the SO mice, the analogous increase was only 6 ± 3% (n = 10). Similar cadmium-induced increases in fecal 45Ca excretion in both OV and SO mice occurred during the seventh and eighth rounds of collection. By comparing the extreme groups in the experiment, OV mice exposed to dietary cadmium (OV/+Cd) excreted twice as much 45Ca into feces as did the SO mice not exposed to cadmium (SO/–Cd).

Effect of Cadmium on Fetal Rat Limb Bones in Culture.

From reports of cadmium levels in the blood of animals exposed to cadmium (2), we estimated the cadmium concentration in the plasma of mice exposed to 50 ppm dietary Cd to be ~10 nM. To determine whether 10 nM Cd could directly increase bone resorption in organ culture, we exposed fetal rat limb bones prelabeled with 45Ca to cadmium in the presence and absence of PTH. As indicated in Table 1, cadmium at 10 nM in the medium stimulated bone resorption to an extent similar to that elicited by 1 nM PTH (experiment 1). In experiment 1, bone resorption in the presence of 10 nM Cd plus 1 nM PTH was no greater than that in the presence of 10 nM Cd alone, perhaps because the rate of bone resorption was already maximal with 10 nM Cd. In contrast, cadmium at a 100-fold higher concentration in the medium (1 μM Cd) did not by itself significantly increase bone resorption in fetal rat limb bones, and it significantly inhibited the effect of PTH (experiment 2).

**DISCUSSION**

Results of our studies show that cadmium stimulated loss of bone mineral to a greater extent in OV mice than in SO controls. After the menopause, when ovarian hormone production ceases, women generally experience an increased loss of bone mass for ~10 years (23, 24). The extent of
Consideration must be given to whether cadmium influenced intestinal calcium absorption in the mice in our study. And Sato et al. (29–31) and Fullmer et al. (32) have shown that oral cadmium in the range of 50 ppm decreases vitamin D-stimulated intestinal calcium absorption and intestinal calcium-binding protein concentrations in young growing rats and chickens. In contrast, Yuhas et al. (33) found an increase in intestinal calcium absorption in adult rats exposed to dietary Cd at 100 ppm. In the mice in our 45Ca study, we measured intestinal calcium absorption in each mouse by the balance method (i.e., by subtracting unabsorbed calcium in feces from calcium in food). [Unabsorbed calcium in feces was obtained by subtracting endogenous calcium excreted in feces from total fecal calcium (34).] Results indicate that Cd at 50 ppm caused no decrease in intestinal calcium absorption in the adult mice in our study; mean values of percentage calcium absorbed during the 96-hr collection periods after the start of cadmium exposure were 15 ± 3%, 27 ± 3%, 26 ± 3%, and 31 ± 2% (mean ± SEM) for mice in the SO/−Cd, SO/+Cd, OV/−Cd, and OV/+Cd groups, respectively. Consequently, the increased release of 45Ca from bone that occurred in the cadmium-exposed mice in our 45Ca study appears not to have been a secondary response to a cadmium-induced decrease in intestinal calcium absorption.

The rapid response to dietary cadmium in vivo (Fig. 4) provides evidence that cadmium may have acted directly on bone. Additional evidence for this hypothesis comes from the striking stimulation of bone resorption that occurred in cultured fetal rat limb bones at 10 nM Cd (Table 1). These results are significant because 10 nM Cd is a concentration in the range that is expected in the circulation of the cadmium-exposed mice in our study. The in vitro effect of cadmium was found to be biphasic, with a much lower bone resorption response at 1 μM Cd, a concentration higher by several orders of magnitude than anticipated in the plasma of cadmium-exposed animals (35). Our small bone resorption response at 1 μM Cd (Table 1), which was not statistically significant in our study, is similar in magnitude to that reported by Miyahara et al. (36, 37) at 10 μM Cd with cultured embryonic chicken bones. In fact, cadmium at the higher micromolar concentrations has been shown to have a number of different effects in in vitro systems: Sakai et al. (38) and Miyahara et al. (39) have shown a cadmium-induced inhibition of bone matrix formation and ossification; we have demonstrated an inhibition of PTH action (Table 1); Cooper et al. (40) have demonstrated an inhibition of electrically stimulated neurotransmitter release at the frog neuromuscular junction. Cd at 1 μM in the latter system (40) appears to act by blocking calcium channels, thereby preventing the influx of calcium that leads to neurotransmitter release. In our study, Cd at 1 μM may also have blocked calcium channels in the cultured fetal rat limb bones and inhibited the influx of calcium that has been shown to initiate the bone resorption response to PTH. The mechanism by which Cd at 10 nM so strikingly stimulated bone resorption in our study, however, still needs to be elucidated by further investigation.

The impact of osteoporosis on the quality of life of our aging population and the need to develop reliable strategies for treating this condition have led to excellent studies to identify differences between osteoporotic and nonosteoporotic individuals (41–44). We have identified one agent, cadmium, that enhances the loss of bone mineral after ovariectomy in mice and, at physiological concentrations in vitro, enhances bone resorption in organ culture. Our results reported here, along with those of others, are consistent with a significant role of cadmium in the etiology of Itai-Itai disease. They also identify postmenopausal women exposed either environmentally or industrially to cadmium as a group with an increased potential to lose bone mineral at an accelerated rate after menopause.
In the general population, cigarette smoking is a significant source of cadmium exposure (5, 7). Cadmium concentrations in whole blood have been shown to be 10–30 nM in smokers compared to only 2 nM in nonsmokers (7). On the basis of data in animals and humans (45, 46), cadmium in blood soon after exposure is present in plasma, whereas it later becomes bound to red blood cells. Consequently, during the smoking process itself, cadmium concentrations in plasma (and possibly saliva) could likely reach 10 nM, a concentration that caused maximal bone resorption in bone organ culture (Table 1). The possibility, therefore, needs to be investigated that increased cadmium exposure combined with decreased estrogen concentrations (8, 9) may play an important role in the lower trabecular bone mineral content of premenopausal women who smoke (10) and in the increased risk of osteoporosis and early tooth loss in postmenopausal women who smoke (11–14).

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