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## Experimental reproduction of itai-itai disease, a chronic cadmium poisoning of humans, in rats and monkeys

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### Abstract

To establish a useful animal model of Itai-Itai disease (IID) of humans, we conducted the following experiments.

Experiment 1 : Toxic effects of Cd were compared between ovariectomized (OX) and non-OX rats after daily, intravenous injection of cadmium (Cd) chloride for 14 days. In this experiment, we demonstrated that OX rats were more susceptible to Cd-induced nephrotoxicity and hepatotoxicity than non-OX rats.

Experiment 2 : OX rats were injected with Cd at doses of 1.0 and 2.0 mg/kg, 5 days a week, for 13 weeks. The bone Cd content was gradually increased for 13 weeks in a dose-dependent manner. Calcium and phosphorus contents in the bone and serum levels of parathyroid hormone and osteocalcin were not significantly different between Cd-treated and control rats. Mild osteomalacic lesions in the cortical bones of the midshaft haversian canals as well as chronic nephropathy appeared in the rats of the 2.0 mg/kg group.

Experiment 3 : OX rats were treated with Cd at doses of 0.5 and 0.05 mg/kg for 70 weeks. The rats of the 0.05 mg/kg group showed slight anemia and mild degeneration of tubular epithelium after 50 weeks of treatment. In the 0.5 mg/kg group, the rats showed definite osteomalacia of bones and nephrosclerosis. The Cd concentration in the bones increased for the first 25 weeks, but was replaced gradually with iron at from 50 to 70 weeks of the administration period. Iron deficiency anemia appeared in the 0.5 mg/kg group at from 12 to 25 weeks, and changed to renal anemia after 50 weeks of administration. The anemia at 50 and 70 weeks was normocytic and normochromic, and serum erythropoietin levels were not elevated in response to the decrease of hemoglobin concentrations of red blood cells.

Experiment 4 : Ten, OX cynomolgus monkeys were given intravenous injections of 0, 1.0 or 2.5 mg/kg/day Cd, 2 or 3 days per week, for 13 to 15 months. Normocytic and normochromic anemia, renal lesions characterized by tubular atrophy and interstitial fibrosis (Cd nephropathy), and bone lesions characterized by an increase of osteoid and osteopenia (Cd osteopathy) were induced in the monkeys treated with Cd.

These results demonstrated that chronic cadmium toxicosis similar to IID of humans was reproducible in rats and monkeys by repeated intravenous injection of Cd and that a disease entity closely resembling IID of humans could be induced in experimental animals by chronic Cd toxicosis without participation of malnutrition, vitamin D deficiency, impaired

absorption at the intestinal mucosa or multiparous birth.

Key words: Cadmium toxicosis, Itai-itai disease, Osteomalacia, Ovariectomy, Renal anemia

### Introduction

Itai-itai means ouch-ouch in English, and human patients affected with the itai-itai disease (IID) complain of severe and continuous pain caused by spontaneous, multiple fractures of bones. The disease was first recognized in Toyama Prefecture, located at the center of the Japanese mainland and facing the Sea of Japan, in 1946, soon after the World War II, by a medical practitioner named Dr. Noboru Hagino. The disease occurred around the Jintsu River and he assumed that some toxic substances included in the water of the river might be the cause. Upstream there was a large mine for lead and zinc.

Cadmium (Cd) was a major by-product of lead and zinc, and was discarded as a useless metal until recently. During World War II, a large amount of zinc had been mined and cadmium was released in the river. Thus, Cd polluted the water, soil, rice, vegetables, humans and animals around the river.

In 1961, Dr. Hagino and his co-workers first presented their hypothesis that the cause of IID was Cd in a scientific meeting. In 1968, the Japanese Government officially acknowledged the disease as the first pollution-related disease caused by an industry-derived pollutant, Cd. After that, many efforts were made to remove Cd from the area: monitoring the waste fluid from the mine and extensive replacement of the polluted soil. The total number of designated victims of the disease was 183 as of January 1999. In the past 10 years, several patients have been newly acknowledged, since Cd once accumulated in the body cannot easily be expelled from the

tissue.

IID occurs mainly in post-menopausal women and characteristic pathological findings of the disease are tubular nephropathy, osteomalacia and renal anemia. Many workers<sup>1,2,10,12,18,20,31,42)</sup> have tried to reproduce the bone lesion and renal anemia by Cd intoxication using various animals, but few of them<sup>36)</sup> have succeeded in producing osteomalacia. Renal anemia has not been observed in Cd-treated animals. Because of the difficulties in the experimental reproduction of the lesions distinctive of IID by Cd treatment, some researchers insist that the real cause of IID is not Cd toxicosis, but malnutrition or vitamin D deficiency. The paucity of animal models of IID has also impeded better understanding of the pathogenesis of tubular nephropathy, renal anemia and osteomalacia of IID, development of novel treatments for the disease, and establishment of more reasonable criteria for the diagnosis of the disease.

In 1990, my co-workers and I started the following experiments to develop an animal model of the disease. Our research has been focused on the reproduction of osteomalacia and renal anemia as well as tubular nephropathy in animal models. Throughout our experiments, we adopted the intravenous route for Cd administration rather than p.o., since the absorption rate of Cd in rats through the intestinal mucosa is 0.4~0.5% and total Cd intake can be accurately calculated in intravenous administration. We inoculated ovariectomized (OX) female rats or OX monkeys with Cd, since IID occurs mainly in postmenopausal women.

Prior to the experiments, we found that

the LD<sub>50</sub> of intravenously administrated Cd in rats was about 5.2 mg/kg in an acute toxic experiment.

## Materials and Methods

### *Experiment 1*<sup>22)</sup>

**Animals and treatment:** A total of 45 female Sprague-Dawley (SD) rats (Charles River Japan, Kanagawa) was obtained at 4 weeks of age. One day later, laparotomy was performed under anesthesia with pentobarbital sodium (50 mg/kg, ip) and the bilateral ovaries were resected completely. One week later, the rats were divided into three groups, two OX groups with Cd administration and a control OX group. Each group consisted of 15 rats and 2-3 animals were housed in a single polycarbonate cage with a barrier system at a temperature of 20-25°C with a relative humidity of 40-70% and a 12-hr light/dark cycle. The animals were allowed free access to rat chow (MF, Oriental Yeast Co., Tokyo) and sterilized water. To provide a comparison, 30 non-OX female rats divided into Cd administration and control groups were housed under the same conditions.

**Experimental protocol:** The rats were injected intravenously with saline (1.0 ml/kg) or CdCl<sub>2</sub> (2.0 and 3.0 mg/kg) on a daily basis for 14 days. Cd solutions were prepared by dissolving pure CdCl<sub>2</sub> (Wako Pure Chemical Industries, Ltd., Tokyo) in 0.9% saline. The Cd-treated non-OX rats were injected with 3.0 mg/kg of Cd. On days 3, 7 and 14 of the experiment, five rats each of the OX and non-OX groups were exsanguinated from the inferior vena cava after blood sampling under anesthesia with pentobarbital sodium. Necropsy was performed and the hematologic, biochemical, and histologic findings for the OX and non-OX rats were compared.

**Cd concentration:** Cd was measured in the frozen liver and kidney tissue from three

rats per group by inductively coupled plasma-atomic emission spectrometry.

**Histopathology:** Tissue samples collected at necropsy were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, cut into 5- $\mu$ m sections, and stained with hematoxylin and eosin (HE) or Giemsa stain. The femur and sternum were decalcified in 10% formic acid-formalin for three days before processing into HE sections.

**Immunohistochemistry:** Two rats (one OX and one non-OX animal) were treated with 3.0 mg/kg of Cd plus an intravenous injection of 100 mg/kg of 5-bromo-2-deoxyuridine (BrdU; Sigma Chemical Co., Tokyo). One hour after the injection, they were euthanized by exsanguination under anesthesia. The kidneys were fixed in absolute ethanol at 4°C overnight and then embedded in paraffin-wax, after which 5- $\mu$ m sections were deparaffinized and dehydrated. Following incubation with 4 N HCl for 30 min at 37°C and rinsing twice for 5 min each in cold boric borate buffer (pH 9.0), the sections were digested with 0.05% pronase E (Sigma Chemical Co., Tokyo) in phosphate-buffered saline (PBS; pH 7.6) for 20 min at 37°C. After two rinses in PBS, a labeled streptavidin-biotin stain (DAKO LSAB Kit, DAKO Co., CA, USA) was applied to the sections. The sections were incubated with the primary anti-BrdU antibody (DAKO Co.) diluted 1:80 in 0.05 M Tris buffer (pH 7.6) for 2 hr at room temperature. After washing, the sections were incubated in 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAKO Co.) in Tris buffer, followed by the addition of 0.02% hydrogen peroxide. Finally, the slides were rinsed and counterstained with HE. BrdU-positive cells in the renal tubules were counted under a X 200 objective, and cell proliferation was calculated using the unit square labeling index (USLI). The size of the renal specimens was measured

by a point-counting method.

*Electron microscopy*: Slices of the cortex of the right kidney from two rats of each group were fixed by immersion in 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate, and were observed using an electron microscope.

*Statistical analysis*: Dunnett's multiple-comparison test was used for evaluation of the data.

#### *Experiment 2<sup>21)</sup>*

*Animals and treatment*: A total of 54 female SD rats were obtained at 4 weeks of age. At the next week, a laparotomy was performed under anesthesia. Two weeks later, the rats were randomly assigned to three groups (two treatment groups and one control group) of 18 rats each.

*Experimental protocol*: The rats were injected in the tail vein with saline (1.0 mg/kg) or CdCl<sub>2</sub> (1.0 and 2.0 mg/kg), 5 days a week, for up to 13 weeks. The concentrations were adjusted on a weekly basis according to body weight. At 4, 8 and 13 weeks after the treatment was commenced, six animals per group were anesthetized and exsanguinated from the inferior vena cava after collecting blood. Necropsy was performed and all macroscopic abnormalities were recorded. The success of ovariectomy was confirmed by the absence of ovaries and uterine atrophy. The right femur was cleaned of soft tissue and the bone length measured with slide calipers. At the final necropsy, the tibias of three rats per group were examined roentgenographically. Blood samples were subjected to biochemical and hematologic investigations. Serum concentrations of calcium (Ca) and inorganic phosphorus (P) measured by an automatic analyzer using the o-cresolphthalein complexone method and phosphomolybdic acid

method, respectively.

*Cd, Ca, P and metallothionein (MT) concentrations*: The Cd contents in frozen tissues of the liver and kidney of three rats per group were analyzed by flame atomic absorption spectrophotometry according to the method of Dudley et al. (1985)<sup>8)</sup>. The humeri of the same rats were subjected to analyses of Cd, Ca, and P. A certain quantity of bone was digested in nitric-hydrochloric acid and the concentrations of these metals were measured by atomic absorption spectrophotometry. MT concentrations in the kidney and liver were measured in three rats of each group by the Cd saturation-hemolysate method. The concentration of MT in each tissue was calculated in units of micrograms of MT per gram, assuming 1 ng atom Cd bound /mol MT.

*Assays for parathyroid hormone and osteocalcin*: Serum concentrations of middle parathyroid hormone (PTH) and osteocalcin (bone glaoprotein, BGP) were determined by RIAs in blood samples of three animals per group at 8 and 13 weeks. These assays were performed using commercially available kits (Rat-PTH-MM, Incstar, Stillwater, MINN, USA, and Rat Osteocalcin RIA, Biomedical Technologies Inc., Stoughton, MA, USA).

*Urinalysis*: Urine was collected on ice from six rats in each group on the day before the termination of the experiment by placing them in individual stainless-steel metabolism cages for 15hr. Total urine volume was recorded and aliquots of the urine were frozen for determination of N-acetyl- $\beta$ -D-glucosaminidase (NAG),  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT), Ca, and creatinine. NAG was assayed using a commercially available kits, the sodium-cresolsulfonphthaleinyl N-acetyl- $\beta$ -D-glucosaminide substrate method (Shionogi Co., Ltd., Osaka) by a spectrophotometer (UV-730; Shimadzu Co.).  $\gamma$ -GT, Ca and creatinine were assayed using commercially available

kits (Iatron Laboratories Inc., Tokyo) by a autoanalyzer.

*Histopathology*: Tissues were collected systematically at necropsy, and processed routinely for histopathological examination. The femur, sternum and lumbar vertebra (L1 to L3) were decalcified in 10% formic acid formalin. The posterior half of the sternum, the ultimate thoracic vertebra (T13) and both tibiae were fixed in 0.5% cyanuric chloride in methanol containing 1% (0.1M) N-methyl morpholine (Yoshiki, 1973)<sup>41)</sup> for 2 days, and decalcified paraffin sections were prepared. Two additional rats treated with 2.0 mg/kg of Cd for 13 weeks and a matched control rat were euthanized by exsanguination under anesthetization. The right proximal tibia and lumbar vertebra (L1) were dissected and fixed in 70% reagent methanol with Villanueva's osteochrome stain<sup>39)</sup>, dehydrated, and embedded in poly-methyl-methacrylate. A longitudinal section of the tibia and transverse section of the vertebra, approximately 300  $\mu$ m in thickness, were made with a diamond cutter (Maruto Instrument Co., Ltd., Tokyo) followed by manual grinding to reduce the thickness to 20  $\mu$ m, and mounted on glass slides.

#### *Experiment 3*<sup>14,15)</sup>

*Experimental animals and treatment*: A total of 54 female SD rats were obtained at 6 weeks of age and the bilateral ovaries were completely excised under anesthesia. Complete disappearance of the estrus cycle was confirmed by vaginal smear testing in all animals. Three weeks later, the rats were randomly assigned to three groups (two treatment groups and one control group), and were administered Cd from 10-weeks of age.

*Experimental design*: The rats were injected in the tail vein with saline (1.0 ml/kg) or CdCl<sub>2</sub> (0.05 and 0.5 mg/kg), 5 days a week, for 50 weeks (control and 0.05 mg/kg

groups) or 70 weeks (0.5 mg/kg group). Prior to the necropsy at 12, 25, 50 and 70 weeks, blood samples were collected from six rats per group and subjected to hematology and blood chemistry examinations.

*Hematology and blood chemistry*: The parameters evaluated for hematology and blood chemistry were erythrocyte count (RBC), hematocrit (Ht), hemoglobin concentration (HB conc.), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count, urea nitrogen (UN), creatinine, iron (NPS method), and total iron binding capacity (TIBC).

*Urinalysis*: Urine samples were collected from six rats of each group at 8, 13, 16, 20, 24, 28, 32, 36, 40, 44 and 49 weeks. Total urine volume was recorded and aliquots of the urine were frozen for determination of urinary NAG, LDH and creatinine levels.

*Serum erythropoietin (EPO) level*: The levels of EPO in the sera of rats sacrificed at 12 and 50 weeks were determined using a commercially available kit (Chugai Pharmaceutical Co. Ltd., Tokyo) by radioimmunoassay.

*Plasma ferritin level and the ferritin content in the liver*: The levels of ferritin in the plasma and liver of rats sacrificed at 25 and 50 weeks were determined by latex immune agglutination nephelometry using a commercially available kit (Iatron Co.).

*Histopathology*: The kidneys, spleen and bones collected at necropsy from all animals were fixed in 10% neutral buffered formalin and processed routinely for histological examination. Berlin blue stain for the demonstration of iron and Goldner stain for osteoid were applied to undecalcified sections of the femur and vertebrae of the rats treated with 0.5 mg/kg of Cd for 70 weeks.

#### *Experiment 4*

*Experimental animals and treatment:* Ten, female cynomolgus monkeys (*Macaca fascicularis*) approximately 5-years old were obtained, and both ovaries of each one were completely excised under anesthesia. One month later, the monkeys were randomly assigned to three groups (two treatment groups and one control group), and were administered Cd (two treatment groups) or saline (control group) for 13 to 15 months.

*Experimental design:* The monkeys were injected in the tail vein with saline (four monkeys), 1.0 (three monkeys) or 2.5 (three monkeys) mg/kg/day of CdCl<sub>2</sub>, 2 or 3 days a week, for 13 to 15 months. Blood and urine samples were collected once a month and submitted to the following examinations. All the animals were killed and necropsied after Cd treatment for 15 months, and one monkey at 2.5 mg/kg group was killed at 13 months, since the animal became moribund.

*Hematology and blood chemistry:* Each blood sample was analyzed for WBC, RBC, Hb, Ht, MCV, MCH, reticulocyte and platelet counts, ASAT, ALAT,  $\gamma$ -GT, LDH, ALP, CK, T-Bil, UN, creatinine, Glu, T-Cho, F-Cho, TG, PL, T-Pro, Alb, A/G, Ca, P, Na, K and Cl.

*Urinalysis:* All samples were analyzed for Glu, Bil, Ket, Pro, Uro, OB, pH, NAG, LDH,  $\gamma$ -GT, ALP and LAP.

*Histopathology:* At necropsy, the liver, kidneys, femur, lumbar vertebrae and sternum were collected, fixed in 10% neutral buffered formalin and processed routinely for HE staining. The bones were decalcified and processed for osteoid staining according to Yoshiki's method<sup>41)</sup>.

## Results

### *Experiment 1*

*Body weight, biochemical data and Cd content:* Both OX and non-OX rats showed temporary excitement with flushing of the

skin at the time of Cd administration. Growth retardation in both treated OX groups was more prominent than that in the non-OX 3.0 mg/kg group. All the rats that received Cd showed mild hypochromic microcytic anemia and there was no difference in severity between the OX and non-OX groups. Biochemistry tests showed that AST and ALT were increased and the total cholesterol level and A/G ratio were decreased on day 14 of Cd administration in both OX and non-OX rats. However, the BUN and creatinine levels showed little change in both OX and non-OX rats. The hepatic and renal concentrations of Cd increased in a dose-dependent manner, and the Cd concentrations in both organs on day 14 were comparable in OX and non-OX groups. Cd was not detected in the control rats.

*Histologic findings:* In Cd-treated OX rats, renal lesions were not found on days 3 and 7. However, both degenerative and regenerative changes of the proximal convoluted tubular epithelium were observed in treated OX rats euthanized on day 14. The incidence and severity of the tubular damage in rats treated with 3.0 mg/kg of Cd was greater than that in those given 2.0 mg/kg. In non-OX rats given 3.0 mg/kg Cd, degenerative and regenerative changes of the tubular epithelium were also observed, but they were mild and less frequent.

Focal hepatocyte necrosis was found in two OX rats treated with 3.0 mg/kg Cd for 3 days. The incidence of the hepatocytic necrosis increased thereafter and all the Cd-treated OX rats showed the lesion on day 14. In contrast, hepatic damage was not found in the non-OX rats. The femur and sternum were unremarkable in all rats.

*Immunohistochemical findings:* In the kidneys of the OX rats, BrdU-labeled cells were frequently observed in the proximal con-

voluted tubules, and labeled cells were also diffusely distributed in the medulla. On the other hand, labeled cells were sparsely distributed throughout the kidneys of the non-OX rats. USLI revealed that the number of proliferating epithelial cells in OX rat kidneys was about 2.5-fold that of non-OX rats, and the increase was especially prominent in the cortex.

*Electron microscopic findings*: Conspicuous pathological changes were observed in the proximal convoluted tubules of OX rats treated with 3.0 mg/kg Cd for 14 days. Non-OX rats given 3.0 mg/kg Cd and OX rats given 2.0 mg/kg Cd also exhibited similar changes, but the incidence was significantly lower than that in the OX rats given 3.0 mg/kg Cd.

#### *Experiment 2*

*Body weight, hematology and biochemistry*: In the 2.0 mg/kg group, growth retardation was continuously prominent from 2 to 13 weeks, and weight loss became marked from 8 weeks. In the 1.0 mg/kg group, the body weight decreased significantly from 10 weeks. The decreases in HT, HB conc., MCV and MCH were significant in the 2.0 mg/kg group at 8 and 13 weeks. Cd administration resulted in a significant increase of total protein with a decrease of the A/G ratio from 4 to 13 weeks. The values of AST and ALT in the 2.0 mg/kg group at 13 weeks were increased 10-fold and 6-fold, respectively, compared to the control. In addition, there were significant increases in serum concentrations of Ca and inorganic P in the 2.0 mg/kg group at 8 and 13 weeks. Rats in the 1.0 mg/kg group showed similar but less conspicuous hematologic and biochemical changes than the 2.0 mg/kg group.

*Cd, MT, Ca and P concentrations of organs*: Hepatic and renal concentrations of Cd and MT increased with dose dependency at 4 weeks, and the concentrations were much dif-

ferent between the 1.0 and 2.0 mg/kg groups at 8 and 13 weeks. The concentrations of Cd and MT were higher in the liver than in the kidney at every week examined. In the control group a basal level of MT was detected, but Cd was absent in both organs. The bone Cd content increased gradually until termination of the experiment. The Ca and P concentrations in the bones of control and Cd-treated rats were not different.

*Urinalysis*: In the course of administration, both water consumption and urine volume increased gradually. The polydipsia and polyuria peaked at about 8 weeks in the 2.0 mg/kg group and subsequently disappeared by the termination of the experiment. In the 1.0 mg/kg group, the symptoms were detected later than in the 2.0 mg/kg group and continued to termination. NAG and  $\gamma$ -GT values in the Cd-treated rats were markedly increased, and urinary excretion of Ca was increased in comparison to the control rats.

*Gross findings*: Mild fibrosis of the liver and foci of scarring in the kidney were detected in the 2.0 mg/kg group at 13 weeks. There was no significant difference in the femur length between control and Cd-treated rats.

*Histopathologic findings*: In the kidneys of Cd-treated rats, vacuolation of proximal convoluted tubular epithelium, single-cell necrosis and/or regeneration of the epithelial cells were observed in the outer cortical zone at 4 weeks. Thereafter, multifocal lesions of tubular atrophy with interstitial fibrosis were distributed throughout this zone. An increased number of coagulating necrotic cells and hypertrophy of collecting tubular epithelium were seen from the inner cortical zone down to the medulla at 13 weeks. However, considerable areas of both the cortex and medulla were intact. The incidence and severity of such changes were higher and more severe

in rats treated with 2.0 mg/kg Cd than in those treated with 1.0 mg/kg Cd.

Bone lesions were restricted mainly to the distal portion of the femur and the proximal portion of the tibia. On the cortical bone of the femoral and tibial midshaft of Cd-administered rats, haversian canals were dilated and surrounded by an increased amount of uncalcified matrix composed of osteoid seams, which were demonstrated by an osteoid stain. Undecalcified sections of the tibial cortical bone showed a moth-eaten or Swiss cheese pattern due to irregular dilatation of the haversian canals with increased osteoid seams. Osteoblasts and osteoclasts were not observed in the dilated canals. The thickness of the cortical bone was almost normal. On the other hand, in the metaphysis of Cd-treated rats cancellous bone mass increased with time. The trabeculae were thickened and cohered to each other and showed increased opacity beneath the metaphyseal plates by roentgenogram. The number of osteoid seams demonstrated by the osteoid stain was increased on the trabecular plate at 13 weeks. The changes present in cancellous and cortical bone were more frequent and more severe in the 2.0 mg/kg group than in the 1.0 mg/kg group. In the 2.0 mg/kg group, a slight increase of osteoid seams was also observed in the trabecular bone of the lumbar vertebrae of two rats at 13 weeks.

Cd-treated rats showed a diffuse increase of hepatocytic eosinophilia and a few foci of single cell necrosis of hepatocytes at 4 weeks. At a later stage, periportal hepatitis was also detected with piecemeal necrosis in the limiting plate, especially in the 2.0 mg/kg group. Mild hypertrophy of parathyroid chief cells was observed in rats treated with Cd for 4 to 13 weeks.

*Hormone assays:* Serum levels of PTH and BGP of three animals per group were not

significantly different between Cd-treated and control groups, with wide deviation of values.

### *Experiment 3*

*General condition and body weight:* The rats in the 0.5 mg/kg group showed temporary excitement at the time of Cd administration from 8 weeks, and this aggressive feature increased with the progression of administration. The water intake increased from 13 weeks, and this increase became marked from 15 weeks. Weight gain was suppressed from 7 weeks, and thereafter body weight was significantly low until termination of the experiment. None of these changes appeared in the 0.05 mg/kg and control groups during the administration period.

*Hematology and blood chemistry:* RBC, Ht and HB Conc. were significantly low in the 0.5mg/kg group from 12 to 50 weeks, and these decreases became marked with time. Reticulocyte counts of the group increased from 12 to 50 weeks. MCV and MCH were decreased at 12 and 25 weeks. At 50 weeks, however, the decreases of MCV and MCH disappeared and, instead, a slight decrease in MCHC was noted. The blood chemical data from the group revealed a decrease in the plasma iron level and an increase in TIBC from 12 to 50 weeks. In addition, elevations of BUN and creatinine were also observed at 50 weeks.

*Urinalysis:* NAG and LDH markedly increased from 16 weeks, and the increase continued until the termination of the experiment in the 0.5 mg/kg group. The urine volume started to increase from 13 weeks, and this increase became marked from 16 weeks in the group.

*Correlation between serum EPO and hemoglobin levels:* In the 0.5 mg/kg group, the serum EPO level significantly increased at 12 weeks, but was not increased at 50 weeks.

The increase of EPO in each rat was proportional to the decrease in hemoglobin at 12 weeks, whereas the EPO was not elevated with the decrease in hemoglobin at 50 weeks.

*Plasma level of ferritin and ferritin content in the liver*: The plasma ferritin level was significantly increased at 25 and 50 weeks in the 0.5 mg/kg group. The ferritin content in the liver was significantly decreased at 12 weeks, but increased at 50 weeks in the 0.5 mg/kg group.

*Macroscopical and histological findings*: The kidneys were macroscopically enlarged at 25 and 50 weeks in the 0.5 mg/kg group. Histologically, mild degeneration, necrosis and regeneration of the tubular epithelium were observed at 12 weeks, but these changes became severe with slight cortical fibrosis and infiltration of lymphocytes at 25 weeks. At 50 weeks, cortical fibrosis, glomerulosclerosis, hypertrophy and hyperplasia of the collecting tubular epithelium with interstitial edema, and dilatation of renal tubules were observed in almost all animals. In the 0.05 mg/kg group, regeneration of the tubular epithelium was slightly observed at 50 weeks.

The femur, tibia, sternum and vertebrae at the 0.5 mg/kg group showed thickening of secondary spongiosa at the metaphyses, dilatation of the haversian canals at the cortex, and increased amounts of osteoid tissue around the bony spicules of the secondary spongiosa and haversian canals. These changes appeared after the administration of Cd for 50 weeks and progressed till the end of the experimental period (70 weeks). Morphometric analysis of the femur and sternum using an image analyzer revealed significant increases of osteoid tissue at 50 and 70 weeks. None of the rats at the 0.05 mg/kg group showed abnormalities of the bone.

#### *Experiment 4*

*Hematology and blood chemistry*: Reduc-

tion of RBC, Hb, and Ht appeared from 6 to 7 months in the 1.0 and 2.5 mg/kg groups. The increases were more prominent in the 2.5 mg/kg group. P decreased from 5 to 7 months in both Cd-treated groups, and the decrease accompanied a decrease of Ca in the 2.5 mg/kg group. Increases of ALAT, ASAT, LDH, ALP, BUN, F-Cho, TG, PL, and Glu were observed in both Cd-treated groups, and decreases of T-Pro and Na appeared in the 2.5 mg/kg group.

*Urinalysis*: NAG, LDH,  $\gamma$ -GT, ALP, LAP and Glu were increased prominently from 4 to 5 months in both Cd-treated groups.

*Histopathology*: Diffuse interstitial fibrosis accompanying atrophy or dilatation of the urinary tubules and hyalin casts were observed in both Cd-treated groups. The femur, lumbar vertebrae and sternum revealed significant increases of osteoid and reduced amounts of cancellous bones.

### Discussion

Cd intoxication has been reported to cause tubular nephropathy characterized by degeneration and regeneration of the convoluted tubular epithelial cells<sup>5,8,11,18,20</sup>. Identical renal lesions were found in Experiment 1. In addition, increased DNA synthesis in the proximal convoluted tubular epithelium was also demonstrated in this study by BrdU labeling.

Although 90% of IID occurs in postmenopausal women, the pathological mechanism leading to this preferential occurrence is unknown. In the present experiment, the concentrations of Cd in the kidney and liver tissue were similar in OX and non-OX rats, but the hepatic and renal lesions were far more severe in OX rats. Therefore, Experiment 1 clearly demonstrated that ovariectomy enhanced Cd-induced nephrotoxicity and hepatotoxicity in rats.

Histologic changes of the kidneys follow-

ing chronic Cd intoxication in Experiment 2 were nephropathy characterized by tubular epithelial degeneration, regeneration, and subsequent tubular atrophy with interstitial fibrosis<sup>8,12</sup>. Such renal tubular atrophy and interstitial fibrosis with clinical polyuria and an increase in enzymuria were reproduced by Cd administration for 13 weeks in Experiment 2.

The skeletal changes of IID have been variously reported as being characterized by severe osteomalacia with multiple pathologic fractures and irregularly increased osteoid seams around trabeculae, and osteoporosis<sup>19</sup>. By experimental Cd intoxication, Takashima et al. (1980)<sup>36</sup> reproduced osteomalacic changes after 19-month Cd intoxication in rats. In Experiment 2, a similar osteomalacic change appeared in cortical bones after 8 to 13 weeks of Cd intoxication in ovariectomized rats. We used ovariectomized rats and injected Cd intravenously. These might be possible reasons for the acceleration of the toxic effect of Cd on the bone<sup>4,32</sup>. The pathogenesis of osteomalacia in IID is still under debate among researchers. In the present study, the renal disturbance observed as clinical polyuria and increased enzymuria accompanied by morphological nephropathy and/or hypertrophy of parathyroid chief cells might have had some effect on the bone lesions induced by Cd. However, an osteomalacic change of the cortical bone appeared in the Cd-treated rats showing accumulation of Cd, normal Ca and P concentrations in bone with hypercalcemia, hyperphosphatemia, and an insignificant deviation from control rats in serum levels of proteins, PTH, and osteocalcin. Focal metaphyseal osteosclerosis was also seen in Cd-treated rats. These findings in Experiment 2 were inconsistent with the hypothesis that osteomalacia develops by an indirect action of Cd through abnormal Ca ho-

meostasis resulting from renal osteodystrophy<sup>18</sup>, malnutrition<sup>7</sup>, secondary hyperparathyroidism<sup>17</sup>, or impaired absorption at the intestinal mucosa<sup>3,24</sup>. Therefore, a direct action of Cd on bone is a possible mechanism for the development of osteomalacia in experimental Cd toxicosis<sup>4,10,32,40,42</sup>. Further evidence for the direct action of Cd was the deposition of Cd on the bone matrix in a dose-dependent manner. This might disturb bone remodeling and mineralization<sup>6,26,35</sup>.

To elucidate the mechanism of anemia in IID, many epidemiological surveys of human IID and animal experiments have been done. However, there is a major discrepancy between the epidemiological surveys and the animal experiments, i.e., anemia in IID patients is renal anemia<sup>16,28,29</sup>, while the anemia in experimental animals is Fe-deficiency anemia<sup>13,33</sup>. In Experiment 3, anemia appeared in the 0.5 mg/kg group at 12 weeks and persisted till the termination of the experiment. The anemia was microcytic and hypochromic at 12 and 25 weeks. It accompanied decreased levels of plasma iron and ferritin content in the liver, and increased levels of plasma iron and TIBC at 12 and/or 25 weeks. The increase of the serum EPO level at 12 weeks was related to the decrease in the hemoglobin concentration. These findings suggested that the anemia observed at 12 and 25 weeks was influenced by iron deficiency. Oral administration of Cd predominantly inhibited the intestinal iron absorption by competing with iron in the iron transport system in the mucosal epithelium of the intestine, and produced bone marrow hyperplasia at the late erythroid progenitor level due to iron deficiency<sup>13,33</sup>. In Experiment 3, however, Cd was injected intravenously, so the mechanism of iron deficiency remains unknown.

At 50 weeks, the microcytic hypochromic erythrocytes disappeared and, instead, nor-

mocytic normochromic erythrocytes were observed. The serum EPO level was not elevated in the rats showing low levels of hemoglobin. The major supplier of the serum EPO is the kidney<sup>9,34)</sup>, and the organ was severely damaged at 50 weeks in the present experiment. Therefore, these findings suggest that the anemia at 50 weeks was not iron-deficient but nephrogenous. The present experiment demonstrates that the renal anemia occurring in IID patients can be experimentally reproduced in the rat by chronic Cd intoxication.

Osteoporosis similar to that of IID patients was reproduced in rats by the administration of Cd for more than 50 weeks in Experiment 3. This confirmed the results of our previous experiment (Experiment 2), in which osteoporotic changes appeared after Cd intoxication for 13 weeks. Cd content in the bone increased for the first 25 weeks, but turned into an abrupt decrease thereafter. In substituting Cd, the iron concentration in the bone increased. Osteoid tissue was the major site of the iron deposition in these bones. Recently, Noda et al.<sup>27)</sup> reported a prominent increase of iron at the mineralization front of the bone of IID patients. Although we do not know the exact mechanism of this substitution of iron for Cd or the significance of the deposition of iron at the mineralization front, osteoporosis accompanying iron deposition in osteoid tissue might be common and significant features of the IID and chronic Cd toxicosis of rats.

In the past three decades, several attempts<sup>3,25,30)</sup> were made to reproduce IID in monkeys but none of them were successful except for inducing renal dysfunction by chronic Cd toxication. In Experiment 4, Cd-intoxicated monkeys showed anemia at from 4 to 5 months with normal levels of MCH, MCV and MCHC; that is, normocytic normochromic

anemia. Elevations of NAG, LDH,  $\gamma$ -GT, ALP and LAP in urine were detected at from 4 to 5 months in Cd-treated monkeys, and these changes accompanied the pathological changes of the kidney identical to cadmium nephropathy such as interstitial fibrosis with tubular atrophy. In the bones of the Cd-treated monkeys, osteomalacic (increase of osteoid tissue) and osteoporotic (atrophy of the cancellous bone) changes coexisted and the quality of bone lesions was in accordance with the Cd osteopathy of humans. In addition, hypophosphatemia appeared from 5 to 7 months. All of these results indicated that we produced an animal model of IID in rats and monkeys, and these animals had almost all the distinctive changes of IID such as hypophosphatemia, osteomalacia with osteoporosis, renal anemia and chronic tubular nephropathy. Our present results clearly indicate that a disease entity closely resembling IID disease of humans can be induced in experimental animals by chronic Cd intoxication without participation of malnutrition, vitamin D deficiency, impaired absorption of the intestinal mucosa or multiparous birth. Our animal model of IID may be beneficial for the following: development of novel therapeutic drugs for IID patients, investigating the pathological mechanism of bone and renal lesions in IID, and establishing more reasonable criteria for the diagnosis of the disease.

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