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Urinary Type I Collagen Cross-Linked N-Telopeptides in Inhabitants 18 Years after Cessation of Exposure to Cadmium in Japan

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The most severe stage of chronic cadmium (Cd) poisoning is known in Japan as "itai-itai" (pain-pain) disease (Nogawa 1981). The natural history of cadmium poisoning includes renal and bone damage. There were several Cd-polluted areas in Japan including the Jinzu River basin in Toyama as well as this study's area of focus, the Kakehashi river basin in Ishikawa prefecture. In the Kakehashi river basin area, Cd contamination was found in 1968. Several epidemiological studies on health effects have since been conducted and contaminated soil has been replaced since 1981(Kido et al. 1990, 1995).

Past studies revealed osteopenia in inhabitants with Cd-induced renal tubular dysfunction using microdensitometer and dose-response relationship analysis (Kido et al. 1991). A significant relationship between Cd exposure and adverse health effects has been reported (Nogawa et al. 1989; Kido et al. 1991, 1995). Renal dysfunctions exposed by Cd mediated bone damage have also been reported on (Aoshima et al. 2003; Kido et al. 1990). In addition, bone Gla-Protein (BGP) and alkaline phosphatase (ALP) which are osteoblastic indicators, are elevated in exposed populations as compared with people in non-polluted areas (Tsuritani 1994).

Furthermore, an assay for the measurement of the bone resorption marker, Type 1 collagen cross-linked N-telopeptides (NTx), was developed by Hanson (Hanson 1992). NTx is produced directly from active osteoclasts at the bone surface. The amount of NTx produced is related to bone resorption activity, such that increased levels of NTx are thought to reflect increased bone resorption by osteoclasts.

Our purpose here was to clarify bone damage by chronic Cd poisoning by measuring urinary NTx levels in a population of Japanese inhabitants 18 years after cessation of exposure to cadmium.

MATERIALS AND METHODS

Inhabitants in one of the most cadmium polluted regions of the Kakehashi River basin in Ishikawa Prefecture, Japan, were selected. The control group lived in a

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non-polluted area about 45km from the exposed population. The control group had never lived in a Cd-polluted area and rarely consumed rice grown in Cd-contaminated areas. Subjects in the control district had health checkups in their district, and were inhabitants who gave their agreement to participate in the study. The Cd-exposed subjects were inhabitants who agreed to the cooperation in the study. Inhabitants older than 50 years old were selected as subjects of study in both groups. 49 men aged 50-87 years old (mean65.2±11.1years), and 57 women aged 50-84 years old (mean 67.1±9.5 years) in the Cd-polluted area and 22 men aged 65-89 years old (mean76.5±5.7 years), and 38 women aged 48-86years old (mean73.7± 8.6 years) in the non-polluted area participated in this study. This study was performed in 1999. Both Cd-exposed and non-exposed subjects with bone lesions such as secondary osteoporosis (along with primary hyperparathyroidism, rheumatoid arthritis, and the taking of medication for the chemical regulation of steroids or calcium) were excluded from the study.

Morning void urine specimens in the exposed group and spot urine samples in the control group were collected in polyethylene bottles. Urinary pH was measured and adjusted to less than 5.8 when necessary. All specimens were kept frozen at -20°C until the analysis could be performed. NTx was analyzed by enzyme immunoassay using a commercial kit (OSTEOMARK, Mochida Pharmaceutical Co., LTD. Japan). The unit of NTx is expressed as nM Bone Collagen Equivalent (BCE)/ mM creatinine. Urinary Cd and Cu concentrations were measured with flameless atomic absorption spectrometry using Hitachi Model Z180-80 after dilution with nitric acid (Kido et al. 1984) and urinary Zn concentration was measured with flame atomic absorption spectrometry using Hitachi Model Z180-80 after acidification with nitric acid and centrifugation. After acidification with hydrochloric acid, Ca and Mg concentrations were measured by flame atomic absorption spectrometry (a Hitachi Model Z180-80) and P concentration was analyzed using the Allen method (Allen et al. 1940). Total protein (TP) was analyzed using a commercial kit (Tonein-TP, Otsuka Pharmacy, Japan), β_2-Microgloblin(β_2-MG) was measured by radioimmunoassay (Pharmacia β_2-micro RIA, Pharmacia Diagnostics AB, Sweden), and *N*-acetyl-β-D –glucosaminidase (NAG) was analyzed using a commercial kit (NAG test Shionogi, Shionogi Pharmaceuticals, Japan). Urinary creatinine concentration was determined using the Jaffe reaction method (Bonsnes and Taussky 1945). Urinary glucose and amino acid concentrations were measured using the o-toluidine boric acid method (Fukushima and Sakamoto 1974) and trinitro benzene sulfonic acid method (Fukushima and Kobayashi 1975).

Values for AminoN, NAG, Glucose (Gl), TP, β_2-MG, Cu, Cd, Zn, Mg, Ca and P were log-normally described and log transformed before statistical analysis. Means between the two groups were compared using Student's *t*-test. Pearson's correlation coefficient was calculated to clarify the relationship among NTx and other indices in the urine samples from the polluted area. Stepwise multiple regression analysis was performed as a dependence variable with NTx and independent variables with age and other urinary index.

RESULTS AND DISCUSSION

Mean age and biological parameters in both sexes of Cd-exposed and non-exposed subjects older than 65 years old are shown in Table 1. In order to achieve consistency in age between the groups, the age of subjects was set at older than 65 years old. Mean urinary β_2- MG, Cd, Ca and P concentrations of Cd-exposed men aged more than 65 years old were significantly higher than those in the corresponding non-exposed men.
Similarly, mean urinary Cd, Mg, Ca and NTx concentrations of Cd- exposed women aged more than 65 years old were significantly higher than those in corresponding non-exposed women (Table1).

Simple correlation coefficients among age and biological parameters in subjects aged more than 65 years and all subjects are shown in Table 2. In men aged more than 65 years, NTx levels only showed significant positive correlation with Mg. In women aged more than 65 years, NTx showed significant positive correlations with aminoN, NAG, Gl, TP, β_2-MG, Cu, Cd, and Ca.

Following stepwise multiple regression analysis, significant multiple correlation coefficients were observed in both sexes. In men, Mg showed significant standard partial regression coefficients with NTx. In women, aminoN and Cd showed significant standard partial regression coefficients with NTx (Table 3).

This study clarified that even 18 years after severe Cd exposure, body burden of Cd was significantly higher in Cd-exposed subjects than in non-exposed subjects using urinary Cd concentration analysis. Cadmium-induced renal tubular dysfunction, a typical critical symptom of toxic cadmium exposure, still existed in some of the Cd-exposed subjects. Preliminary research also showed the presence of many tubular disorders in Cd-exposed subjects who were over 65 years of age (Sunaga et al, 2003). In addition, a sex difference in NTx value was observed. In women significant difference was shown between Cd-exposed and non-exposed subjects as measured by NTx levels, used as a bone resorption marker. There was association between NTx and Cd, or aminoN concentrations in women by multiple regression analysis. In women bone resorption characterized by increased osteoclast cell activity is influenced by estrogen and so the risk of osteoporosis in post-menopausal woman became high.

NTx concentrations measured in women over 60 years of age from a former Cd-polluted area in Japan were higher than those reported by Baba for healthy Japanese women who were 70-80 years old and post-menopausal (Baba 1999). As a result, we were thought that elevated Cd concentrations in the exposed subjects chronically accelerated bone resorption almost two decades after the last exposure. This was postulated as being a result of the long half-life of cadmium (T1/2=10-30 years) whose deleterious effects can persist even when the acute exposure is removed (Friberg 1985).

Table 1. Results of urinary analysis in Cd –exposed and non-exposed subjects over 65 age.

	Cd-exposed				Non-exposed				*P*-value
	N	Mean	S.D.	Range	N	Mean	S.D.	Range	
Men									
Age (year) §	27	73.5	7.57	65-87	22	76.5	5.67	65-89	
β_2-MG (μg/g cr.)	27	239	17.0	0.9-24446	22	53.4	3.58	6.4-737	*
Cd (μg/g cr.)	27	4.01	1.91	1.6-11.8	22	1.84	1.56	0.1-3.5	**
Mg (mg/g cr.)	27	69.4	2.01	29.5-893	22	42.6	6.17	29.5-152	
Ca (mg/g cr.)	27	111	1.69	46.0-328	22	68.2	1.81	20.0-202	**
P (mg/g cr.)	27	579	1.35	268-997	22	438	1.66	133-854	*
NTx (nM BCE/nM cr.) §	26	33.2	17.8	9.3-74.6	20	26.0	1.66	6.6-65.7	
Women									
Age (year) §	34	73.4	6.19	65-84	38	73.7	8.62	67-86	
β_2-MG (μg/g cr.)	34	336	11.1	0.2-94836	32	229	2.81	39.8-2321	
Cd (μg/g cr.)	34	6.03	2.37	1.6-186	35	2.86	1.82	0.6-8.6	**
Mg (mg/g cr.)	34	94.7	1.76	19.9-416	37	52.6	4.92	6.1-390	*
Ca (mg/g cr.)	34	157	1.66	52.1-413	37	109	1.79	24.7-369	**
P (mg/g cr.)	34	638	1.31	339-1157	37	552	1.56	184-1014	
NTx (nM BCE/nM cr.) §	34	73.1	39.9	14.4-174	35	37.3	17.2	10.6-81.3	**

§Arithmetic mean and standerd devision(S.D.). Other values are expressed as geometric mean and geometric S.D.

* Significant difference ($P<0.05$) between Cd-exposed and non-exposed subjects.

** Significant difference ($P<0.01$) between Cd-exposed and non-exposed subjects.

Table 2. Simple correlation coefficients in Cd-exposed subjects over 65 and total age.

Men	Total n=49	Age[§]	aminoN	NAG	Gl	TP	β_2-MG	Cu	Cd	Zn	Mg	Ca	P	NTx
	Age (year) [§]		0.18	0.39**	0.08	0.53**	0.60**	0.51**	0.48**	-0.04	0.34 *	0.15	-0.12	0.29
	aminoN (mg/g cr.)	0.39 *		0.38**	0.64**	0.45**	0.42**	0.31 *	0.17	0.02	0.48**	0.67**	0.44**	0.34
	NAG (U/g cr.)	0.61**	0.40 *		0.32 *	0.68**	0.52**	0.35 *	0.11	0.18	0.24	0.20	-0.24	0.27
	Glucose (mg/g cr.)	0.42 *	0.58**	0.38		0.43**	0.24	0.15	0.04	0.17	0.33 *	0.35 *	0.31 *	0.09
over	Total Protein (mg/g cr.)	0.58**	0.55**	0.74**	0.55**		0.76**	0.60**	0.40**	0.10	0.30 *	0.39**	-0.02	0.27
65age	β_2-MG (μg/g cr.)	0.44 *	0.52**	0.59**	0.37	0.83**		0.60**	0.51**	-0.02	0.34 *	0.41**	0.10	0.32
	Cu (μg/g cr.)	0.17	0.63**	0.36	0.48 *	0.73**	0.68**		0.70**	0.04	0.20	0.37**	-0.02	0.23
n=27	Cd (μg/g cr.)	0.04	0.44 *	0.10	0.33	0.42 *	0.51**	0.51**		-0.10	0.15	0.31 *	0.01	0.02
	Zn (mg/g cr.)	-0.21	-0.12	0.08	0.06	0.05	-0.06	0.03	-0.16		0.06	-0.14	-0.10	0.36
	Mg (mg/g cr.)	0.38 *	0.55**	0.24	0.36	0.31	0.36	0.19	0.17	-0.03		0.62**	0.17	0.59**
	Ca (mg/g cr.)	0.19	0.64**	0.19	0.30	0.45 *	0.49 *	0.47 *	0.46 *	-0.28	0.65**		0.35 *	0.21
	P (mg/g cr.)	-0.22	0.36	-0.35	-0.01	-0.07	0.11	0.20	0.21	-0.29	0.28	0.43 *		0.07
	NTx (nM BCE/nM cr.)[§]	0.09	0.06	0.13	-0.06	0.18	0.10	0.18	0.15	0.15	0.38**	0.03	-0.06	
Woman	Total n=57	Age[§]	aminoN	NAG	Gl	TP	β2-MG	Cu	Cd	Zn	Mg	Ca	P	NTx
	Age (year) [§]		0.26	0.46**	0.24	0.48**	0.35**	0.32 *	0.20	-1.00	0.12	0.17	-0.15	-0.01
	aminoN (mg/g cr.)	-0.05		0.41**	0.59**	0.65**	0.57**	0.66**	0.44**	-0.34	0.27 *	0.40**	0.20	0.47**
	NAG (U/g cr.)	0.26	0.41 *		0.19	0.63**	0.40**	0.51**	0.44**	-0.13	0.17	0.18	-0.19	0.47**
	Glucose (mg/g cr.)	0.08	0.79**	0.38 *		0.41**	0.36**	0.50**	0.29 *	0.00	0.16	0.15	-0.03	0.31
over	Total Protein (mg/g cr.)	0.29	0.62**	0.63**	0.63**		0.67**	0.74**	0.52**	-0.18	0.16	0.25	0.04	0.49**
65age	β_2-MG (μg/g cr.)	-0.11	0.63**	0.40 *	0.67**	0.69**		0.55**	0.41**	-0.10	0.10	0.15	0.00	0.52**
	Cu (μg/g cr.)	0.16	0.66**	0.57**	0.65**	0.82**	0.66**		0.67**	-0.09	0.17	0.22	-0.1	0.47**
n=34	Cd (μg/g cr.)	0.02	0.46**	0.56**	0.44**	0.65**	0.63**	0.75**		0.01	0.22	0.13	-0.03	0.58**
	Zn (mg/g cr.)	-0.22	-0.03	-0.22	-0.10	-0.30	-0.27	-0.18	0.00		-0.05	-0.18	0.19	-0.27
	Mg (mg/g cr.)	-0.18	0.63**	0.16	0.49**	0.36 *	0.42 *	0.44 *	0.28	-0.02		0.34**	-0.01	0.40 *
	Ca (mg/g cr.)	0.06	0.38 *	0.17	0.18	0.16	0.13	0.31	0.18	-0.19	0.45**		0.16	0.37 *
	P (mg/g cr.)	-0.18	0.19	-0.14	-0.12	-0.01	0.07	-0.14	-0.09	0.25	0.38 *	0.18		0.14
	NTx (nM BCE/nM cr.)[§]	0.11	0.48**	0.34 *	0.29 *	0.45**	0.45**	0.42**	0.48**	-0.16	0.14	0.30 *	0.16	

* significant (P<0.05) ** significant (P<0.01)

[§] Arithmetic mean and Standard Deviation (S.D.). Other values are expressed as geometric mean and geometric S.D.

Table 3. Biological parameters associated with urinary NTx in Cd-exposed subjects selected by step wise regression analysis.

	Men				Women		
	N	β			N	β	
Age §	47	-0.045		Age §	57	-0.047	
aminoN	47	-0.157		aminoN	57	0.327	**
NAG	47	0.043		NAG	57	0.072	
Glucose	47	-0.212		Glucose	57	0.005	
Total Protein	47	0.071		Total Protein	57	0.119	
β_2-MG	47	-0.034		β_2-MG	57	0.195	
Cu	47	0.099		Cu	57	-0.049	
Cd	47	0.085		Cd	57	0.339	**
Zn	47	0.123		Zn	57	-0.151	
Mg	47	0.378	**	Mg	57	-0.022	
Ca	47	-0.338		Ca	57	0.144	
P	47	-0.131		P	57	0.106	
R		0.378	**	*R*		0.564	**

β, standardized partial regression coefficients

R, multiple correlation coefficients.

§ Arithmetic mean and Standard Deviation (S.D.). Other values are expressed as geometric mean and geometric S.D.

* significant ($P<0.05$) ** significant ($P<0.01$)

It was suggested that accumulation of Cd induces renal dysfunction first and slowly advances to increased bone damage through accelerated oscteoclast activity in this study.

Kondo et al. (2003) first suggested the measurement of urinary NTx concentrations in patients with impaired renal tubular function in cadmium poisoning. They reported that NTx concentrations in these subjects were significantly higher than those of common Japanese women of the same average age, including patients with what is known as "itai-itai" disease. We did not collect the recommended second urine sample in the morning because bone metabolism increases NTx in urine at night and levels progressively decrease over the day. This was confirmed by Kondo et al. (2003) who demonstrated stable values of NTx in the daytime. In this study, therefore, NTx levels could be compared in the subjects while these samples were collected both in the morning and daytime.

In this study, we measured bone damage in chronic Cd poisoning using urinary NTx levels. Cd mediated bone damage and renal dysfunctions were reported by Aoshima (2003), who focused on the most severe stage of chronic Cd poisoning in Japan. However we determined the NTx level in the patients who suffered from chronic Cd poisoning in low to moderately – exposed areas. This result suggests that the urinary NTx might be useful to show the risk for Cd-induced bone damage at earlier stage.

Our data showed that for all the women who have lived in a former Cd-polluted area, it is necessary to protect against future health disturbances in those with cadmium poisoning and follow-up studies should be continued in the future.

In men, there was no significant difference in NTx observed between both groups. Men did not show a similar increase in osseous resorption as observed in women. Although a significant relationship between Mg and NTx was indicated, the role of Mg in osseous resorption is unclear and so further research is needed.

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