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Increase of Urinary and Serum Hydroxyproline in Subjects Exposed to Cadmium

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Itai-itai disease (I disease) is characterized mainly by renal tubular damage and osteomalacia accompanied by osteoporosis in subjects with long-term ingestion of excessive cadmium (Cd) (Ishizaki et al. 1968). Most of the studies on the osteopathies of this disease have focussed on mineral metabolism (Nakagawa 1960). For a better understanding of the osteopathies of I disease, we have been interested in collagen metabolism in relation to that of minerals. Although Iguchi and Sano (1975) reported on urinary hydroxyproline (Hyp), an amino acid rich in the collagen of bone, and its significance in patients with I disease, there have not been so many studies on this subject. After this report, high urinary concentration of Hyp in patients with I disease was confirmed and the correlation between increased urinary Hyp and renal reabsorption damage was demonstrated (Nishino et al. 1978 ; Kobayashi et al. 1981). In addition to renal disorders, an increased urinary concentration of Hyp is observed among patients with osteopathies (Bolzonella et al. 1984). Therefore, it is possible that the increased urinary concentration of Hyp may be associated with the osteopathies of patients with I disease. To provide more information about the increased urinary concentration resulting from Cd exposure the measurement of serum concentration of Hyp was also carried out in the present study.

MATERIALS AND METHODS

The Cd-exposed group consisted of 12 female I disease patients (mean age of 72 years, 60 – 84 years) and 13 female so-called I disease observation patients (mean age of 78 years, 70 – 88 years) with renotubular damage but without the typical bone disorder (Shiroishi et al. 1977). A Bed-rest group included in the study consisted of 19 female osteoporosis patients(mean age of 82 years,

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70 – 93 years) who had been staying in N hospital for 3 months or more. The Control group consisted of 24 female patients (mean age of 76 years, 67 – 90 years) who suffered from ischialgia, hypertension, atherosclerosis etc., but otherwise were not impeded from living a normal life.

Two-hour urinary specimens were obtained in the morning and in some cases twenty-four-hour urinary specimens were collected. Blood samples were drawn from subjects before breakfast between 9 and 10 o'clock in the morning of the day when urinary specimens were collected.

Total hydroxyproline (T Hyp) obtained after hydrolysis of the sample and free hydroxyproline (F Hyp) before hydrolysis were determined by colorimetry following the urinary hydroxyproline measurement method of Ikeda et al.(1985). For the measurement of serum F Hyp, 1 ml of serum deproteinised by the addition of 50 μ l of 60 % sulfosalicylic acid was applied to high performance liquid chromatography (HPLC, NIHONBUNKO, TRI ROTAR-V). Serum T Hyp was determined by colorimetry as for the aforementioned urinary measurement. In the present study, the value obtained after subtraction of F Hyp from T Hyp, designated as (T-F) Hyp, was regarded as that which represents peptide-type and protein-bound hydroxyproline in the serum (Le Roy and Keplan 1964). Furthermore they have pointed out that the plasma concentration of peptide-type hydroxyproline only changes under the disorders involving collagen metabolism which could induce changes in (T-F) Hyp concentration. According to Shelton et al.(1972), protein-bound hydroxyproline was related to the Clq component of complement (molecular mass, 410000). So protein-bound hydroxyproline does not appear in urine because of its high molecular weights of proteins. Therefore (T-F) Hyp was defined as peptide-type hydroxyproline (P Hyp) for urine specimen in the present study. The correlation coefficient between values measured by colorimetry (A) and HPLC (B) was 0.94 ($p<0.001$) and the regression line was ; $A = 0.936\ B + 1.24$.

Other serum chemical measurements undertaken included alkaline phosphatase (Al-P, assayed by modified Bessey-Lowry method), inorganic phosphorus (I-P, phosphomolybdate method), calcium (Ca, o-cresolphthalein complexone method), creatinine (Cre, Jaffe method) and urea nitrogen (Urea N, glutamic dehydrogenase method).In urine I-P, Cre and β_2-microglobulin (β_2-m, latex immunoassay method) were analysed. Serum and urine measurements were performed in a Technicon RA-1000 (USA). In order to determine creatinine clearance (Ccre) and tubular reabsorption of phosphorus (%TRP), the urinary and serum specimens aforementioned were used.

Statistical analysis of the results was performed by a t-test or F-test.

RESULTS AND DISCUSSION

The laboratory data of the Cd-exposed and Bed-rest groups compared with the Control group are presented in Table 1. With respect to Al-P, Cre and Urea N, the serum concentrations were significantly higher in both I disease and Observation patients than in the Control group. For these variables no significant difference was observed between the I disease and Observation patients or between

Table 1. Laboratory Data of the Cd-exposed and Bed-rest Groups
(Arithmetic mean ± S.D.)

	Cd-exposed group		Bed-rest group	Control group
	Itai-itai disease patients	Observation patients		
N	12	13	19	24
Age	72 ± 7	78 ± 5	82 ± 7	76 ± 7
Serum Al-P(B.L.U)	4.2 ± 2.4*	4.6 ± 2.8*	2.5 ± 1.0	2.2 ± 0.7
I-P (mg/dl)	3.1 ± 0.8	3.1 ± 0.9	3.8 ± 0.5	3.6 ± 0.4
Ca (mg/dl)	9.4 ± 0.9	9.0 ± 0.9	9.0 ± 0.4	9.2 ± 0.4
Cre (mg/dl)	3.5 ± 1.3***	2.9 ± 1.4***	0.8 ± 0.3	0.9 ± 0.2
Urea N (mg/dl)	36.7 ± 14.8**	28.9 ± 13.7*	15.4 ± 7.0	18.0 ± 5.8
Ccre (ml/min)	12.5 ± 7.3***	14.5 ± 9.5***	47.0 ± 28.1	52.4 ± 30.7
%TRP (%)	47.0 ± 14.2***	48.2 ± 22.3***	89.1 ± 6.6	88.6 ± 5.3
Urine β_2-m(μg/gCre) ‡	112000*** (40800~309000)	107000*** (40700~282000)	2400*** (91~63000)	437 (66~2880)

‡ Geometric mean (Min~Max)
*. **. *** p<0.05, p<0.01, p<0.001, respectively, compared with control group

Table 2. Urinary Concentration and Excretion Rate of Hydroxyproline

			Cd-exposed group			Bed-rest group			Control group		
			N	G.M.	(G.S.D.)	N	G.M.	(G.S.D.)	N	G.M.	(G.S.D.)
F	Hyp	μg/ml		9.4	(2.0) a)		0.6	(3.7)		0.5	(3.9)
		mg/gCre	24	26.7	(2.2) a)	7	2.1	(2.9)	2	5.3	(1.2)
		μg/hr		446	(1.9) a)		33.8	(3.3)		52.8	(4.6)
P	Hyp	μg/ml		14.3	(1.5)**. a)		24.4	(1.5)		20.8	(1.4)
		mg/gCre	25	39.8	(1.5) a)	19	80.2	(1.5)*	24	40.4	(1.5)
		μg/hr		726	(1.5) a)		1384	(1.9)		977	(1.8)
T	Hyp	μg/ml		25.0	(1.5)		20.9	(1.4)		24.6	(1.6)
		mg/gCre	25	70.5	(1.6)***	19	81.5	(1.5)***	24	41.7	(1.6)
		μg/hr		1253	(1.5)		1406	(1.9)*		973	(1.8)

G.M. Geometric mean, G.S.D. Geometric standard deviation
N Number of samples detected, a) p<0.001, compared with bed-rest group
*. **. *** p<0.05, p<0.01, p<0.001, respectively, compared with control group

the Bed-rest and Control groups. The urinary β_2-m concentration was significantly higher in the two subgroups of Cd-exposed patients and the Bed-rest group than in the Control group.

With respect to the urinary F Hyp, 2 out of 24 cases (8%) were positive (0.1 μ g/ml or higher) in the Control group. Twenty four out of 25 cases (96%) in the Cd-exposed group and 7 out of 19 cases (37%) in the Bed-rest group were positive. These positive rates were significantly higher than that of the Control group ($p<0.001$ and $p<0.05$, respectively). Between the former two groups, the Cd-exposed group showed significantly higher value than the Bed-rest group ($p<0.001$). For urinary F Hyp, geometric means of positive cases only are given in Table 2. Both the concentration (μ g/ml or mg/gCre) and the excretion rate (μ g/hr) of the Cd-exposed group were significantly higher ($p<0.001$, respectively) than those of the Bed-rest group whose values were similar to those of the Control group. Only in the Cd-exposed group the concentration (mg/gCre) of F Hyp was correlated with T Hyp ($r=0.70$, $p<0.001$). Therefore it is possible that the increased concentration of T Hyp may be dependent on the increase of F Hyp in the Cd-exposed group.

As shown in Table 2, with respect to urinary P Hyp in the present study, the geometric means of the three variables aforementioned as for concentration were significantly lower in the Cd-exposed group than in the Bed-rest group ($p<0.001$). No significant differences, however, were observed between the Cd-exposed group and Control group, except for concentration designated as μ g/ml. In comparison to the Control group, urinary P Hyp (μ g/ml) in the Cd-exposed group was significantly lower ($p<0.01$) and P Hyp (mg/gCre) in the Bed-rest group was significantly higher ($p<0.05$). From the stand point of creatinine-adjusted concentration, a common indicator of urinary concentration, it may be suggested that the Cd-exposed group excreted a similar amount of urinary P Hyp to that of the Control group, while the Bed-rest group excreted more and resulted in an increase in urinary T Hyp (mg/gCre and μ g/hr) (Table 2). Although Sano et al.(1974) has reported the increase in the concentration of urinary P Hyp of I disease patients, it might be partly explained by the difference in the stage of the disorder. More data would be required to reach a definite conclusion about this.

As given in Table 3, the geometric mean of the serum concentration of F Hyp was significantly higher in the Cd-exposed group compared with that of the Bed-rest ($p<0.01$) and Control groups ($p<0.001$). There was no significant difference between the latter 2 groups. To date, although some investigators have reported serum concent-

Table 3. Serum Concentration of Hydroxyproline (μg/ml)

	Cd-exposed group			Bed-rest group			Control group		
	N	G.M.	(G.S.D.)	N	G.M.	(G.S.D.)	N	G.M.	(G.S.D.)
F Hyp	25	2.5	(0.2)$^{***, a)}$	19	1.7	(0.2)	24	1.5	(0.2)
(T-F) Hyp	17#	3.4	(0.2)$^{b)}$	18#	4.4	(0.2)**	22#	3.5	(0.2)
T Hyp	17#	6.0	(0.2)*	18#	6.0	(0.2)**	22#	5.1	(0.2)

N Number of samples examined
G.M. Geometric mean, G.S.D. Geometric standard deviation
*, **, *** $p<0.05$, $p<0.01$, $p<0.001$, respectively, compared with control group
a), b) $p<0.01$, $p<0.05$, respectively, compared with bed-rest group
For 8 specimens of Cd-exposed group, 1 specimen of bed-rest group and 2 specimens of control group, the data of T Hyp were not available

ration of amino acids among I disease patients (Hoshino et al. 1974), there is not much information about hydroxyproline, and an increase in F Hyp concentration has not yet been confirmed. Hoshino and Tuchiya (1975) reported non-detectable levels of F Hyp in I disease patients with less than 3 mg/dl plasma creatinine. In the present study, since there were some severe cases with more than 3 mg/dl serum creatinine, it was suspected that reduced glomerular filtration might cause an increase in the F Hyp concentration in the serum. However, the lack of a significant correlation between the serum concentration of F Hyp and the glomerular function parameters such as Cre, Ccre and Urea N (data not shown) did not support our speculation.

As shown in Table 3, the mean value of (T-F) Hyp of the Bed-rest group was significantly higher in comparison to that of the Cd-exposed and Control groups ($p<0.05$ and $p<0.01$, respectively). Between the latter two groups there was no significant difference. In general, changes in serum concentration of either F Hyp or (T-F) Hyp have not necessarily been observed in patients with osteoporosis (Aloia et al. 1983 , Minisola et al. 1985). Nevertheless the observation that three healthy males aged 21 – 22 years subjected to prolonged bed rest for 36 weeks showed a decrease in bone mineral components and also an increase in the urinary concentration of calcium and hydroxyproline (Donaldson et al. 1970) is along a same line with the present observation showing an increased serum concentration of (T-F) Hyp in the Bed-rest group. Depending on the change in the serum concentration of either F Hyp or (T-F) Hyp, the T Hyp concentration also changed in respective groups. That is, the increase for the Cd-exposed

Table 4. Correlation Coefficients between Urinary and Serum Concentrations of Hydroxyproline

	Serum F Hyp vs. Urine F Hyp	Serum (T-F) Hyp vs. Urine P Hyp	Serum T Hyp vs. Urine T Hyp
Cd-exposed group	0.422* (N=24)	0.592 (N=17)*	0.629** (N=17)
Bed-rest group	0.444 (N=7)	-0.069 (N=18)	-0.237 (N=18)
Control group	--- (N=2)	0.323 (N=22)	0.343 (N=22)

N Number of samples detected, * $p<0.05$, ** $p<0.01$

group depended on the increase in F Hyp (the correlation coefficient between serum concentrations of T Hyp and F Hyp was 0.78, $p<0.001$), and that for the Bed-rest group on the increase in (T-F) Hyp (the correlation coefficient between serum concentrations of T Hyp and (T-F) Hyp was 0.80, $p<0.001$).

The correlation coefficient between urinary and serum concentrations of hydroxyproline for positive cases in each group is presented in Table 4. For F Hyp, (T-F) Hyp and T Hyp, there were significant positive correlations in the Cd-exposed group but not in the Bed-rest and Control groups. These correlations especially with regard to F Hyp whose increase in both urine and serum is characteristic in the Cd-exposed group suggest that its increased urinary concentration is due to not only reduced renotubular absorption caused by Cd-induced damage (Kobayashi et al. 1981) but also to its increased serum concentration. Iguchi et al.(1982) has demonstrated that Cd inhibits the activity of lysyl oxidase in bone tissue which is involved in the cross-linkage of collagen. The inhibition of lysyl oxidase activity is followed by immature collagen production which is quite labile to dissolution (Jasin and Ziff 1962, Pinnell and Martin 1968). Therefore the present authors speculate that one reason for the increased serum F Hyp in the Cd-exposed group is the accelerated dissolution of immature collagen. According to previous findings on hydroxyproline metabolism, the serum concentration of F Hyp is also regulated by hepatic hydroxyproline oxidase activity ; this enzyme converts F Hyp to pyrroline-3-hydroxy-5-carboxylic acid. Therefore, inhibition of this enzyme activity could possibly result in the increased serum concentration of F Hyp (Varghese et al. 1981). It has been reported that Cd may inhibit the activity of proline oxidase (Sano and Iguchi 1974). Hydroxyproline oxidase which belongs to the group of proline oxidase may also be inhibited by Cd. This process of disturbed amino acid metabolism is another

possible explanation for the increased serum concentration of F Hyp in the Cd-exposed group.

In conclusion, increased concentrations of both urine and serum F Hyp in the Cd-exposed group were considered to be associated with disorders of collagen and/or amino acid metabolism caused by Cd exposure. On the other hand, it is suggested that for the Bed-rest group the increased concentrations of urine and serum (T-F) hydroxyproline may depend on the release of peptide-type hydroxyproline from bone matrix under the absorption process.

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