Proceedings of

NIMD Forum 2005

Joint Workshop on Health Effects of Methylmercury and Cadmium

Current Problems in Risk Evaluation and Risk Management of Methylmercury and Cadmium

12, 13 January, 2005

Venue: Minamata Disease Archives, Conference Hall
55-10 Myojin, Minamata City, Kumamoto 867-0055, Japan

National Institute for Minamata Disease
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Preface

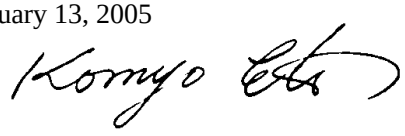
This Forum will be held every year in order to discuss plans for future research subjects in our institute with researchers from foreign countries. We desire to promote productive communication between the guests of other countries and the members of our institute. NIMD Forum has been held annually since 1999. The theme of this year is “Current Problems in Risk Evaluation and Risk Management of Methylmercury and Cadmium”. Up to the present, the problems of the effects of low-dose methylmercury were discussed in the NIMD Forum. Previous themes included “Japan-US Workshop on Human Health Effects of Low Dose Methylmercury Exposure” cosponsored by US-EPA in 2001 and “The Study of Fetal Methylmercury Exposure and Children Development” in both 2002 and 2003. Risk assessments of methylmercury and cadmium were discussed in the 61st meeting of JECFA (Joint FAO/WHO Expert Committee on Food and Additives), Rome, 10-19, June 2003.

This meeting is intended to open discussions between researchers in the fields of methylmercury and cadmium. Such discussions are very important since Japanese people take methylmercury through sea products, fish and shellfish, and also are contaminated with cadmium through rice.

I am very happy to have three presenters from the U.S.A. as co-hosts for this meeting today. I want to express my heartfelt appreciation to Dr. Deborah C. Rice, Dr. Susan L. Schantz and Dr. Christopher De Rosa. Thank you very much for the three doctors’ attendance at this meeting. They have traveled from far a way in spite their busy lives in the world. As Japanese speakers at this meeting, we asked Dr. Fujio Kayama, Dr. Teruhiko Kido, Dr. Rie Masho and Dr. Koji Nogawa. They have graciously accepted to attend the meeting for the presentation of papers. Also, I express my heartfelt appreciation to Dr. Chiharu Tohyama, National Institute for Environmental Studies, for his planning of the agenda. Furthermore, I feel great pleasure to listen to an honorary lecture from Professor Makoto Futatsuka, Kumamoto University, who continues to be a chief research coordinator of the National Institute for Minamata Disease.

Thanks again to all members who have cooperated so that this NIMD Forum could be held today. I hope future communication among researchers in the U.S.A. and Japan will be promoted through this meeting. Also I expect to develop future studies on the knowledge of the effects of heavy metals getting from this meeting among the Japanese researchers. I hope this meeting will produce fruitful results in the future.

January 13, 2005



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Honorary Lecture

Joint Workshop on Health Effects of Methylmercury and Cadmium

Symptoms and Patients

Health and Environment

[Honorary Lecture]

LONG TERM FOLLOW UP STUDY OF HEALTH STATUS IN THE POPULATION LIVING IN A METHYLMERCURY POLLUTED AREA

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Introduction

In addition to the typical features of methylmercury poisoning, previous studies found that a variety of nonneurological symptoms and complaints among Minamata disease patients also differed as compared with the control population. Especially, so-called chronic Minamata disease (chronic methylmercury poisoning) has atypical and subclinical features unlike classical Minamata disease. Moreover, aging, complications accompanying aging, and socio-psychological factors probably modified these clinical features of methylmercury poisoning. The complexity of the facts surrounding the methylmercury poisoning in the Minamata area led to difficulties in diagnosing Minamata disease clinically and in solving the social problems related to assisting the victim.

At present it is important to follow up on the health condition of inhabitants living in the methylmercury polluted area surrounding Minamata City, paying attention to disorders not only of the central nervous system, which have been concentrically studied over 20 years, but to other health issue as well.

Study population and study design

The authors have performed annual follow-up multiple health examinations on about 1,500 persons over 40 years old in Tsunagi Town near Minamata City each summer from 1984 to 2004. Case-control studies were designed to estimate the role of risk factors for these diseases by using geographical differences to compare the verified patients.

Tsunagi Town is located in the southern part of Kumamoto prefecture, next to Minamata City, and faces the Yatsushiro Sea. It had a population of about 5,800 in 1995, about 330 of whom were certified as Minamata disease patients. Although the number of Minamata disease patients in this town is the second largest next to Minamata City, the number as a percentage of the total population is the largest. We analyzed the geographical distribution of the subjects because about 90% of the total number of Minamata disease patients in this locality have

lived in fishing villages where much fish and shellfish were consumed, so it is practical to divide the locality into fishing villages as the polluted area and others as the internal control area.

Based on the mercury content in hair samples collected in these areas in 1960, distribution of estimated intake level of methylmercury was estimated. The proportional rate of intake of toxic doses of methylmercury (0.4mg) was 20% among the inhabitants living in coastal districts.

Results and Discussion

1. Prevalence of some clinical diseases in the population

It is important to follow up on the health status of inhabitants living in the methylmercury-polluted area surrounding Minamata City, paying particular attention to diseases not only of the central nervous system but also of other organs. We have been carrying out such concentric studies for more than 10 years. We have previously studied the cause-specific standard mortality ratios in Minamata disease patients and reported that the SMRs for liver disease and renal disease were significantly raised in male and female patients, respectively. It was also found that complications arising from diabetes could be due to the large number of old people among the autopsy cases. The next step was to clarify the actual prevalence and incidence of liver disease, renal disease, and diabetes mellitus epidemiologically among the population in this area. The aim of this study was to determine the actual prevalence of these diseases and complaints, and to investigate the contribution of various risk factors to these diseases in this area. The study was a population-based cross-sectional mass screening survey. A case-control study was designed to estimate the role of various risk factors including methylmercury exposure for these diseases. Data concerning liver disease, renal disease, and diabetes mellitus were collected on the basis of urine, hematological, physical, and ultrasonographic examinations. Data on risk factors and subjective complaints were collected by interview and other measures. The prevalence of these diseases was not higher in this methylmercury-polluted area compared with other areas in Japan, contrary to what was expected based on standard mortality ratios and pathological findings. There were no positive correlations between those diseases and methylmercury exposure.

2. An analysis of subjective complaints in the population

The aim of this study is to document subjective complaints, to analyze the structure of a population living in a methylmercury-polluted area, and to investigate the relationship between the subjective complaints and methylmercury pollution. A total of 1,304 adults in the polluted area, and 446 age-matched adults in a nonpolluted area were interviewed.

Comparison of prevalence, factor analysis, and cluster analysis were conducted using data drawn from a questionnaire survey about 64 subjective complaints. The population in the polluted area had more various complaints than that living in the nonpolluted area. The factor analysis proposed four factors: nonspecific, sensory arthritic, and muscular. Each of the four factor scores was significantly higher in the population in the polluted area. Subjects who had more complaints were classified into three clusters using cluster analysis. It is possible that not only neurological subjective complaints but also nonspecific complaints of the population in the polluted area might be influenced by past methylmercury exposure.

3. Logistic model analysis of neurological findings in the population

To establish a statistical diagnostic method to identify patients with Minamata disease (MD) considering factors of aging and sex, we analyzed the neurological findings in MD patients, inhabitants in a methylmercury polluted (MP) area, and inhabitants in a non-MP area.

We compared the neurological findings in MD patients and inhabitants aged more than 40 years in the non-MP area. Based on the different frequencies of the neurological signs in the two groups, we devised the following formula to calculate the predicting index for MD: predicting index = $1/(1+e^{-\chi}\times 100$ (The value of χ was calculated using the regression coefficients of each neurological finding obtained from logistic analysis. The index 100 indicated MD, and 0, non-MD).

Using this method, we found that 100% of male and 98% of female patients with MD (95 cases) gave predicting indices higher than 95. Five percent of the aged inhabitants in the MP area (598 inhabitants) and 0.2% of those in the non-MP area (558 inhabitants) gave predicting indices of 50 or higher.

Our statistical diagnostic method for MD was useful in distinguishing MD patients from healthy elders based on their neurological findings.

4. Mental health condition in the population

Little attention has been paid to the mental health of inhabitants of methylmercury-polluted areas in Japan. This study examined the relationship between one's experience with Minamata disease (MD) (such as compensation issues) and psychological distress. The subjects were 133(44.2%) of the 301 inhabitants over the age of 40 years living in two fishing village districts along the coast of the Yatsushiro Sea which had been contaminated with methylmercury. Data on the inhabitants' experience with MD, social network factor, health condition and mental health were obtained using questionnaires including the General Health Questionnaire (GHQ)-30. The proportional odds model was used to estimate the adjusted odds ratios of factors associated with a higher GHQ score after adjustment for age, sex and village. MD status based on MD compensation, level of participation in MD patients' groups, and presence of certified MD patients in the family were significantly associated with

psychological distress. Although these associations decreased after further adjustments were made taking health condition into consideration, MD status, participation in several sit-ins and the presence of certified MD patients in the family maintained marginally positive association with psychological distress. Further investigations with more precise and detailed measurements are needed to corroborate the relationship between inhabitants' experience with MD and mental health.

Conclusion

We conducted a long term follow up study (1984-2004) on the health status of the population (total number of 3,000) in a methylmercury polluted area, Tsunagi Town, Kumamoto Prefecture, Japan.

The results of the study may be summarized as follows:

(1) There were no significant differences in the prevalence of disease structure (ex. liver diseases, renal diseases, diabetes mellitus, cardiovascular diseases).

(2) Subjective complaints which were related to not only neurological but also general complaints were consistently much more common than in the control areas.

(3) Five percent of the inhabitants who were not certified as MD patients gave a high predicting index of MD. They could be affected by methylmercury poisoning. It is important to conduct differential diagnosis.

(4) It is important to take into consideration mental distress not only from physical effects but also from the secondary social damage experienced through Minamata disease in these areas.

[WS-1]

A REVIEW OF 33 CASES WITH *ITAI-ITAI* DISEASE (CADMIUM-INDUCED RENAL TUBULAR OSTEOMALACIA) RECOGNIZED OFFICIALLY FOR THE LAST DECADE

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Itai-itai disease is well known as a health hazard induced by cadmium in the cadmium-polluted areas of the Jinzu River basin in Toyama Prefecture, Japan. In this area, the river was contaminated by slag from a mine upstream, and furthermore, the soil of rice paddies was polluted with heavy metals through irrigation water. Water from the Jinzu River had been used not only for agriculture, but also for household use and drinking. Consequently, because of contaminated water and rice, chronic cadmium poisoning, that is called *Itai-itai* disease, broke out among the people who lived there.

The main clinical features of *Itai-itai* disease are osteomalacia accompanied with osteporosis, and multiple proximal renal tubular dysfunctions. Administrative treatment of the recognition of *Itai-itai* disease by the government of Toyama Prefecture has been in operation since December 1967 for making compensation for her/his illness. A patient who satisfies all the following conditions from I to IV items is recognized as having *Itai-itai* disease. I) The patient should have been residing in an area heavily contaminated by cadmium, and should have been exposed to cadmium. II) The following findings in items III and IV should not be congenital, and should have developed after one's prime (mainly after menopause for females). III) Disorders in renal tubules should be observed. IV) Findings of osteomalacia accompanied with osteoporosis should be evident from X-ray examination or biopsy or autopsy of bones.

As of November 24, 2004, a total of 188 patients (185 women and 3 men) had been recognized as having *Itai-itai* disease. Of these 188 patients, 33 cases (all women) were recognized between 1992 to 2004. The present paper describes the review of these 33 cases on their clinical courses including laboratory, X-ray and autopsy findings to clarify the recent clinical features of Itai-itai disease.

Patients themselves must make application for insisting on recognition as *Itai-itai* disease patients to the Governor of Toyama Prefecture under the law. However, in actuality physician diagnoses her/him as *Itai-itai* disease first and suggests it to her/him. Of the 33 cases, 30 visited Hagino Hospital and only three cases consulted other medical clinics.

The year of birth of these 33 cases ranged from 1898 to 1925. The year of the first application for recognition as *Itai-itai* disease patients to the Governor of Toyama Prefecture

ranged from 1976 to 2004; 11 cases in 1976 to 1984 (Group I), 15 in 1985 to 1994 (Group II), and 7 in 1995 to 2004 (Group III). The average of age at the time of their first applications was 74.0 years old in Group I, 79.1 in Group II and 86.7 in Group III, showing the later applicants were older. The duration between the first application and the recognition as *Itai-itai* disease patients ranged from two months to 17 years with average of 5.8 years.

Nineteen cases (58%) were recognized as having *Itai-itai* disease based on the pathological findings of autopsy after death. Of the 19 cases, 13 were recognized as *Itai-itai* disease patients based on the notice dated April 28, 1993, issued by the Environmental Agency of Japan. Entitled “On the diagnosis of osteomalacia in the recognition of *Itai-itai* disease,” the notice directed that histopathological findings of bone, including those using the Yoshiki method, should be employed in the diagnosis of *Itai-itai* disease, and that the cases screened in the past which had not been recognized as those of *Itai-itai* disease, even though histopathological findings of bone that had been submitted should be critically reviewed again.

The Yoshiki method is a method of staining osteoid matrix, published by Professor Shusaku Yoshiki in 1973. Since osteomalacia is a disorder involving failure of bone calcification, uncalcified bone, i.e., osteoid matrix, increases. Therefore histological identification of osteoid matrix is necessary in the diagnosis of osteomalacia. Using the Yoshiki method, osteoid matrix is intensively stained with eosin. Therefore, this method has superior characteristics, such as easy distinction of osteoid matrix from calcified bone, compared to the previously adopted method.

The remaining 14 cases were recognized during their lifetime, but only 2 cases are alive. The degrees of osteomalacia of these 14 cases were serious as confirmed through clinical symptoms, and biochemical and X-ray findings without bone biopsy.

[WS-2]

THE TEMPORAL PROFILE OF NEUROLOGICAL FINDINGS IN MINAMATA DISEASE - MULTIPLE LOGISTIC REGRESSION ANALYSIS COMPARED WITH THE RESIDENTS IN A METHYLMERCURY-UNPOLLUTED AREA -

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Fifty years have passed since the official discovery of Minamata disease (MD) in 1956. The core symptoms and signs of MD have been considered to be 'glove-and-stocking type' sensory disturbances, ataxia, concentric constriction of visual fields, and hearing impairment. Of 2,265 certified patients with MD in Kumamoto and Kagoshima, 2/3 are already dead. In the survivors, the majority of patients belong to chronic MD which showed an insidious onset after 1960 with mild or vague neurological manifestation, and pathological findings have been modified by aging and various complications. Concerning the temporal profile of neurological findings in MD, there have been neurosymptomatologic studies on the prevalence rate of each symptom and sign but no detailed studies on the possible correlation among neurologic findings or weight. To clarify differences in the severity of MD, a statistical method by calculating discriminants in patients certified as MD is needed to be developed.

In this study, we established such method to clarify the possibility and the severity of MD. Using this established method, we also carried out a survey of neurological findings and complications in certified survivors to clarify the present status.

Using discriminants by multiple logistic analysis that were obtained in certified patients in Kumamoto, Kagoshima, and Niigata, the temporal profile in the severity of MD from the time of certification to the survey in 2003 and the influences of complications were evaluated. Then, a follow-up survey was conducted to evaluate the present neurological symptoms and signs, the status of complications, and ADL in patients in the Minamata and Izumi district who were being treated on an outpatient basis in the Minamata City General Hospital and Medical Center. The subjects were consisted of 3 typical MD patients with the core MD symptoms who survived the period of acute methylmercury intoxication before 1960, 21 chronic MD patients who showed an insidious onset after 1960 with mild and vague neurological symptoms, and 1 fetal MD patient. In addition, data obtained in this survey were compared with neurological findings obtained from examination records/data in the

Minamata Disease Victims Certification Council at the time of recognition about 20 years earlier, and the temporal profile was evaluated. In the 32 outpatients (mean age, 71 yrs) of the Minamata City Medical Center, cerebral infarction was the most frequent complication, being observed in 13 patients. As other complications, cerebellar degeneration (late cerebellar cortical atrophy: LCCA) was present in 1 patient (age, 76 yrs; 62 yrs at the onset after recognition), and HTLV-I-associated myelopathy (HAM) in 2 (ages, 67 and 75 yrs). In the 25 patients (mean age, 71.3 yrs) for whom neurological symptoms and signs at the time of certification could be clarified based on data in the Certification Council or examination records, changes in the discriminant score were evaluated. The mean duration between the time of certification and this survey in 2003 was 25.5 ± 6.6 yrs.

First, changes in the discriminant score obtained by the first method were evaluated. The first Kumamoto method showed a discriminant score of ≥ 80 at the time of certification in 24 patients, after excluding 1 certified in Kagoshima Prefecture, but its decrease in most patients at the time of the survey in 2003 (median value: $99.998 \rightarrow 35.427$). When changes in the discriminant score in each patient were evaluated, only 1 patient showed increases in the discriminant score by all of the first Kumamoto method. Patient A had LCCA as a complication.

Next, changes in the discriminant score were evaluated by the second method obtained from the core symptoms and signs of MD. At the time of recognition, the mean discriminant score was 70.483 ± 38.082 by the Kumamoto method, showing marked dispersion (standard deviation, 34-43). The discriminant score had decreased at the time of the survey in 2003 in most patients, which was similar to the results of the first method (median value: Kumamoto method, $94.770 \rightarrow 5.364$). The third method is based on only 'glove-and-stocking type' sensory disturbances . The discriminant score at the time of certification by the third Kumamoto method was ≤ 10 in 2 patients certified in Kagoshima. At the time of the survey in 2003, the discriminant score had decreased in all patients, being < 20 (median value: $99.637 \rightarrow 0.254$). Our discriminants were obtained in certified patients in Kumamoto, Kagoshima, and Niigata using inhabitants in seaside area similar to Minamata district but not polluted by methylmercury as controls. The temporal profile in the severity of MD from the time of certification to the survey in 2003 and the influences of complications were evaluated. In addition, the status of complications was investigated, and the present status of patients with chronic MD 50 years after discovery was clarified. The mean age of the certified patients with MD in Minamata and Izumi district as the subjects was 70 years, showing the aging of patients with MD. Frequent disorders among the aged such as cerebral infarction, cervical spondylosis, cervico-omo-brachial syndrome, and spondylosis deformans were often observed, and the pathological findings of MD had been complicatedly modified by complications.

The probability of MD was evaluated using the discriminants established in this study. Because many patients with the most severe symptoms and signs with acute methylmercury

intoxication died before our survey, only patients of lower severity might be included in this survey. In spite of such inevitable selection bias in our study design, the discriminant score was generally high by each method at the time of certification but had decreased in most patients at the time of the survey in 2003. This suggested alleviation of the symptoms and signs of MD after a course of about 25 years.

Evaluation of the age at the time of examination and the distribution of discriminant scores suggested changes in the pathological condition of MD at the age of 45 years. At the age of ≤ 45 years, the discriminant score was close to 100. At the age of > 45 years, the discriminant score indicated that "The probability of MD is poor." One factor may be the modification of the pathological condition due to an age-associated increase in the incidence of complications.

Among the subjects in this study, a patient with typical MD was compared with a patient with fetal MD. The discriminant score in the patient with fetal MD did not change from the time of certification to the survey in 2003. In contrast, the patient with typical MD (certified at the age of 47 years) showed alleviation of symptoms and signs. It is unclear whether these findings reflect the development of irreversible neuronal damage induced by methylmercury exposure during the fetal period, or whether symptoms decrease after the age of 45 years even in the presence of fetal MD. Consideration should also be given to the time of methylmercury exposure (fetal period or after birth, childhood or adulthood), and the duration and amount of exposure. In patients with fetal MD, further continuous evaluation of neurological findings is necessary.

When the third Kumamoto method based only on 'glove-and-stocking type' sensory disturbances was used to evaluate the probability and severity of MD, all patients showed a very low discriminant score (< 20) at the time of survey in 2003. This suggests that the differentiation of chronic Minamata disease due to methylmercury based only on sensory disturbances is difficult. We believe that the precise diagnosis of MD based on the distribution of sensory disturbances is essentially unachievable, especially in the aged patients.

[WS-3]

UNDERLYSING MECHANISMS FOR TWO-POINT DISCRIMINATION IN HUMANS

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1. Central mechanisms for two-point discrimination in humans.

We studied the cognitive mechanisms for two-point discrimination (TPD) in 11 normal subjects, using electrical pulses [1]. We used six ball-shaped electrodes placed in line on the dorsal surface of the left hand (Figure 1), and two-point was stimulated by two electrodes randomly selected. We measured the reaction time for TPD and calculated the percentage of correct responses for each two-point stimulation (Figure 2). The subjects' response was significantly affected by the preceding stimuli as well as the distance of the stimuli: for a two-point stimulus condition, subjects tended to feel the stimuli as two-point when the distance between the stimuli was longer than that of preceding stimuli, whereas they felt the stimuli as one-point when the distance was shorter than that of the preceding stimuli (Figure 3). The present results indicate that the TPD process involved evaluation of the distance between the stimuli relatively to that of the preceding stimuli, as well as evaluation of absolute distance between the stimuli.

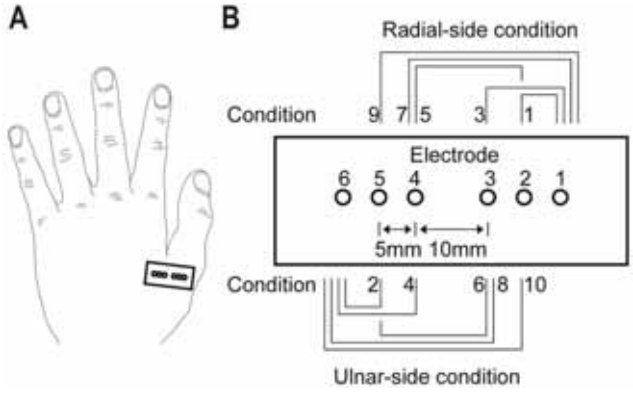

Figure 1: (A) Stimulus electrodes were placed in the radial nerve territory on the dorsal surface of the left hand. (B) Each electrode was placed with a 5-mm distance except the two in the center of the area separated by 10 mm. The combination of electrodes in each stimulus condition was numbered from 1 to 10. For example, condition 1 represents the stimulation applied to electrodes 1 and 2. Conditions 1, 3, 5, 7, and 9 were classified into the radial-side condition, and the others into the ulnar-side condition. (Adopted from reference [1])

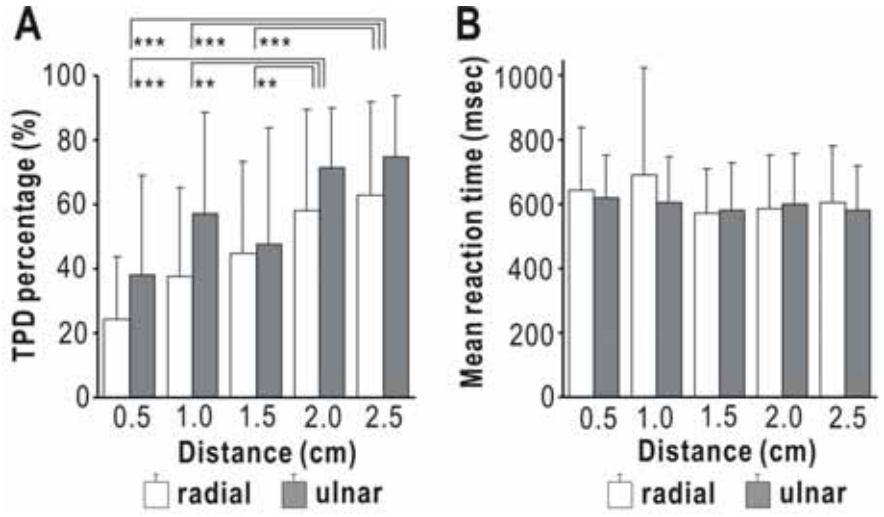

Figure 2: (A) The mean values (± S.D.) of the TPD percentage in each condition are shown. A two-way factorial ANOVA and post-hoc test revealed a significant effect of the distance between electrodes on the TPD percentage. There was no effect of the stimulated side (open column; radial side, solid column; ulnar side). $^{**}p < 0.01$, $^{***}p < 0.001$, Bonferroni-Dunn correction. (B) The mean values (± S.D.) of the reaction time in each condition are shown. There was no significant difference of the reaction time among the conditions or between the stimulated sides. (Adopted from reference [1])

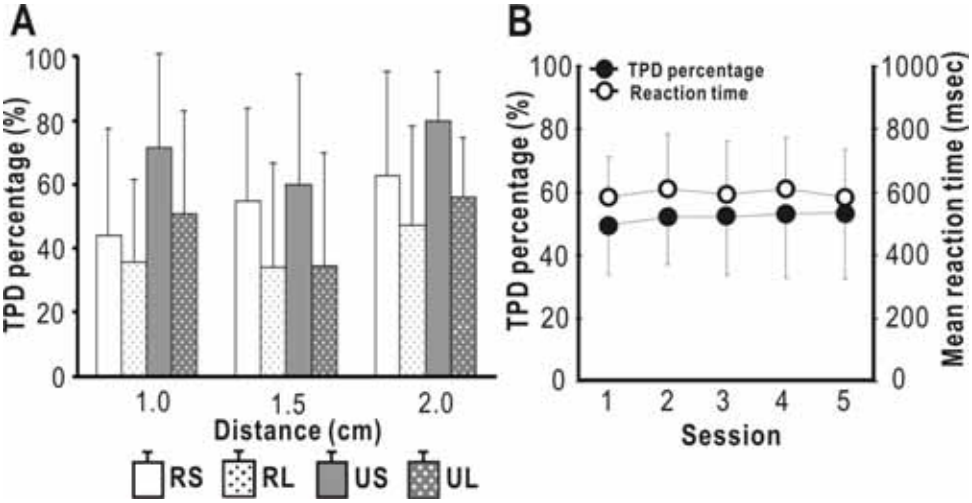

Figure 3: (A) The mean values (± S.D.) of the TPD percentage in each condition are shown: radial side stimulation preceded by longer (RL) and shorter (RS) distance stimulus, and ulnar side stimulation preceded by longer (UL) and shorter (US) distance stimulus. A three-way factorial ANOVA revealed that there were significant effects of both preceding stimuli and distance on the TPD percentage, whereas the effect of side and any interaction were not significant among three factors. Stimuli with the distances of 0.5 and 2.5 cm were excluded from the analysis, since there was no preceding stimulus shorter than 0.5 cm or longer than 2.5 cm. (B) The mean values (± S.D.) of the TPD percentage (●) and the reaction time (○) in each session are shown. There was no effect of session on the TPD performance. Session 1 contains the first 100 successive two-point stimuli; session 2, stimuli 101-200; session 3, stimuli 201-300; session 4, stimuli 301-400; session 5; stimuli 401-500. (Adopted from reference [1])

2. Cognitive processes in two-point discrimination: an ERP study.

We recoded event-related brain potentials (ERPs) to elucidate the temporal features of the cognitive process in TPD [2]. We measured ERPs in 9 subjects during the TPD task, in

which we provided a pair of electrical pulses simultaneously, altering the distance between the electrodes. We analyzed the TPD-related ERPs and investigated the relationship between the potentials and the subjects' judgments. During the TPD task, a negative potential approximately 140 ms after the stimulation (N140) was enhanced as compared to a stimulus counting task (Figure 4). Two late positive components, LPC-1 and LPC-2, whose peak latencies were 300 and 500 ms, respectively, were identified only in the TPD task (Figure 4). The LPC-1 was recorded dominantly in the fronto-central area, while the LPC-2 was detected dominantly in the centro-parietal area (Figure 4). The amplitude of the LPC-2 was significantly modulated by the degree of consistency in the subjects' judgment (Figure 5). On the other hand, these ERP components did not show significant difference between the alternate judgments, i.e. 'one-point' or 'two-point' judgment (Figure 6). Therefore, in conclusion, our results suggest that the N140 is related to the attention toward the stimulation. The LPC-1 and LPC-2 are likely to correspond to the processes represented by P3a and P3b, based on their temporal and spatial behavior.

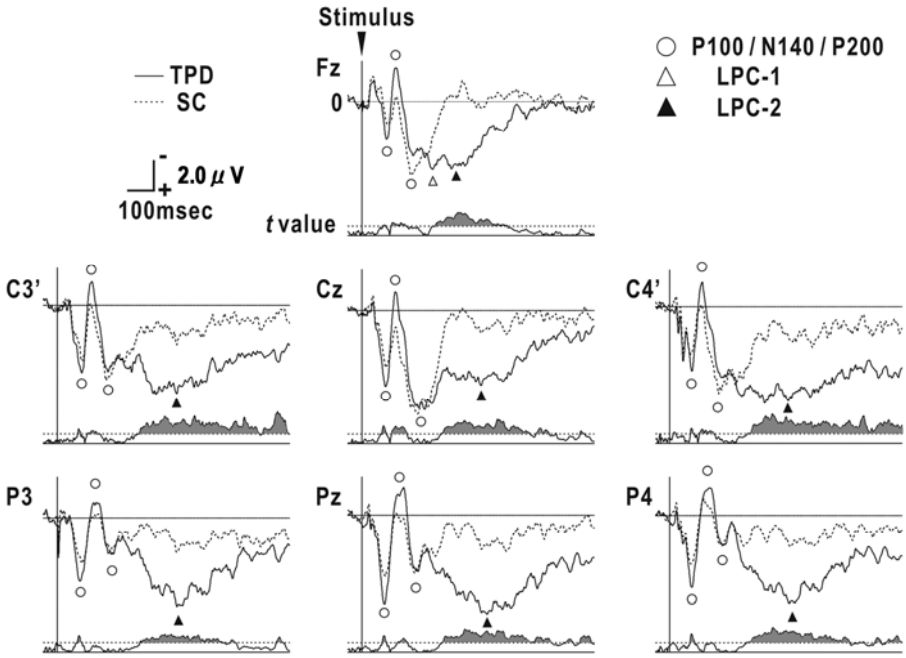

Figure 4: Grand-averaged waveforms obtained from nine subjects during the TPD and SC tasks.
All waveforms were obtained under stimulus conditions with a distance of 1.5 cm. The early components, comprising a positive peak (P100), negative peak (N140) and successive positive peak (P200), were clearly recognized at each electrode in both task sequences. A positive deflection was identified approximately 300 msec after the stimulation only in the TPD condition (LPCs). The LPCs recorded at Fz showed two separate peaks, LPC-1 and LPC-2. The bottom solid line in each graph indicates a *t*-value at each sampling point in the paired *t*-test between the waveforms in the TPD and SC sequences, and the horizontal dotted line indicates a *t* value of 2.11, which corresponds to a *p* value of 0.05. Areas with shadow indicate the period in which the difference was significant. (Adopted from reference [2])

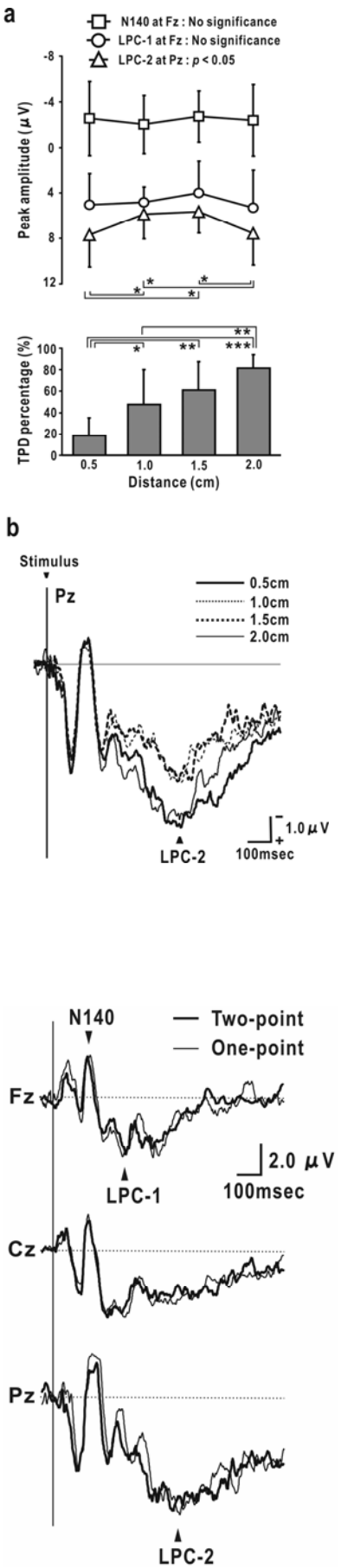

Figure 5: The relationship between the TPD-related potentials and the performance of the subjects.
(a) TPD percentage in each distance and peak amplitudes of corresponding TPD-related ERP components are shown. There was a significant effect of distance between the electrodes on the TPD percentage. On the other hand, a significant effect of distance was found on the peak amplitudes of LPC-2, while there was no significant effect on those of N140 and LPC-1. When the subjects answered "two points" with a higher or lower TPD percentage, the LPC-2 amplitude was significantly larger than that in the stimulus condition with moderate values of TPD percentage.
(b) Grand-averaged waveforms at Pz obtained from nine subjects following the stimulation with various distances between the electrodes. The LPC-2 amplitudes with the distance of 0.5 cm and 2.0 cm were significantly larger than those with 1.0 cm and 1.5 cm. There was no difference in peak latency among the stimulus conditions. (Adopted from reference [2])

Figure 6: The relationship between the TPD-related potentials and the judgement .
Grand-averaged waveforms obtained from seven subjects in each judgment were shown. There was no significant difference in TPD-related potentials between "one point" and "two points" judgments. (Adopted from reference [2])

3. Temporal discrimination threshold on various parts of the body.

The temporal discrimination threshold (TDT) of various parts of the body was investigated in 35 healthy volunteers, and the effect of aging on the TDT was studied in 80 subjects (aged 18-82 years) [3]. Ascending (ATDT) and descending (DTDT) TDT values were measured in 13 areas using a pair of electrical stimuli. Both ATDT and DTDT differed significantly among the body parts ($P < 0.01$, one-way repeated ANOVA), and the TDT was shortest on the index finger and longest on the lower leg, where it was approximately 156% of that on the finger (Figure 7). There was no difference of the TDT value with gender or between sides. There was no effect of aging on the TDT in subjects aged 18-64 years, but the value was prolonged in subjects over 65 years (Figure 8). We suggest that the TDT difference among body parts is mainly due to the difference in sensory processes in the central nervous system, and that it may provide information about changes in the system related to aging.

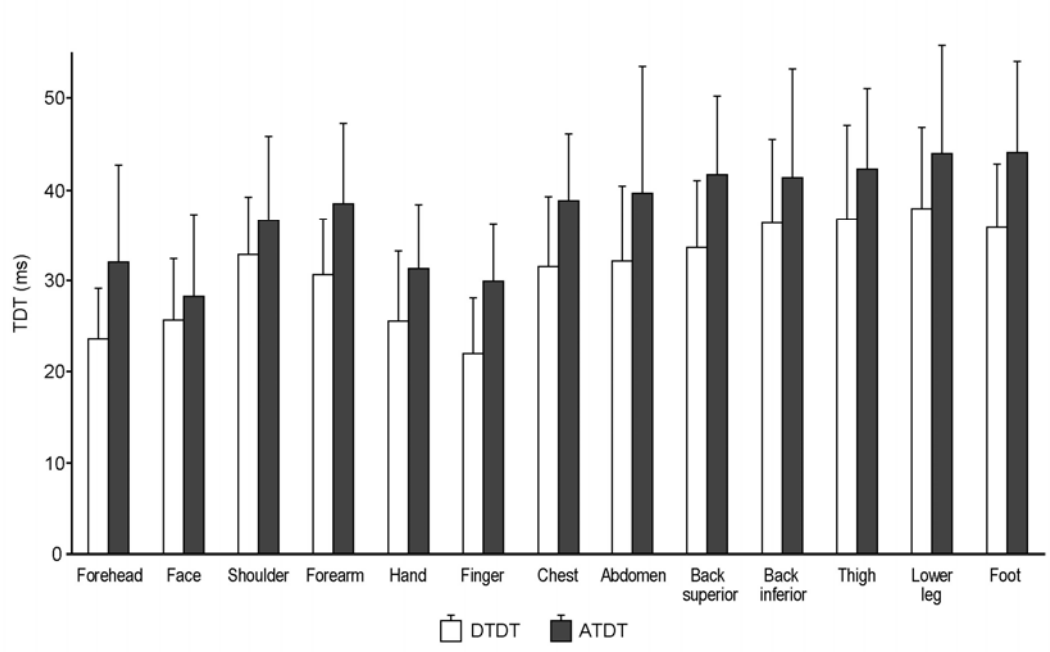

Figure 7: Temporal discrimination threshold (TDT) of various parts on the body. The descending TDT (white columns) and ascending TDT (black columns) values were significantly different among the body parts ($p<0.01$, ANOVA). Each vertical line indicates a standard deviation. (Adopted from reference [3]).

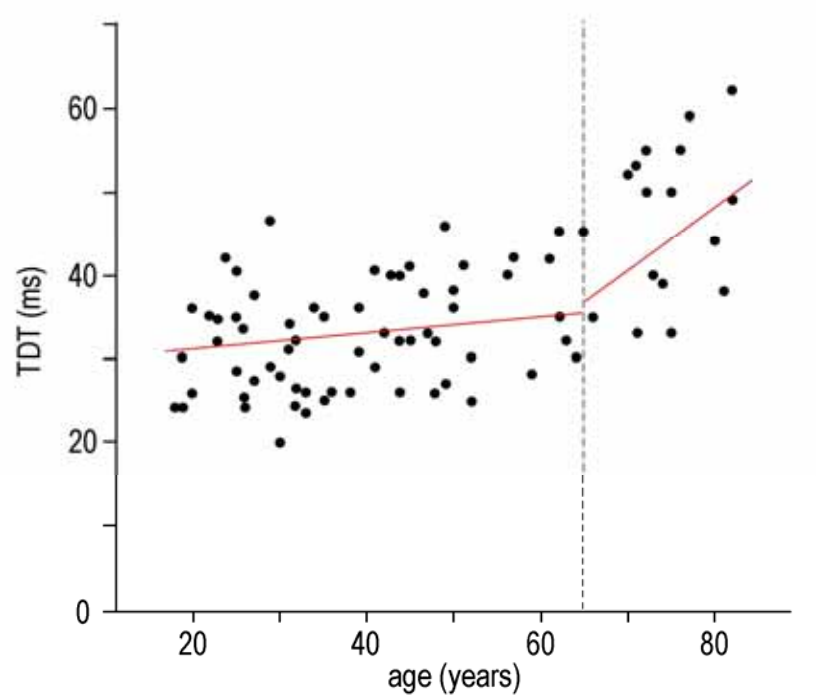

Figure 8: The mean temporal discrimination threshold (TDT) values of descending TDT and ascending TDT on the right index finger in 80 subjects (age range: 18-82 years). In the subjects aged 65 years and over, the TDT value prolonged with aging (Spearman's correlation test, $p<0.001$, Fig. 2). The vertical dashed line indicates age of 65 years. (Adopted from reference [3])

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METHYLMERCURY EXPOSURE IN CURRENT JAPANESE: ESTIMATION FROM HAIR ANALYSIS

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SUMMARY

Methylmercury (MeHg) is an environmental pollutant with neurotoxic effects on the central nervous system. The major exposure route of MeHg to humans is via consumption of fish and shellfish which accumulate the chemical through the food web in an aquatic environment. Hair mercury level is an excellent marker for MeHg exposure. We have been conducting a survey on hair mercury contents among general populations from 14 districts to estimate the current Japanese MeHg exposure level. Total mercury levels of all hair samples collected (12923 in total) were analyzed by the oxygen combustion-gold amalgamation method using an atomic absorption mercury detector. Multiple regression analysis revealed that mercury levels were significantly correlated with several covariates, such as sex, age, the amount of daily intake of total fish/shellfish, a preference for certain fish such as tuna or bonito, and artificial waving. The geometric means for the population without artificial waving were 2.47 and 1.65 μg/g for males (n = 5623) and females (n = 3470), respectively. Hair mercury levels varied with age, and the variations were more significant in males. Since the difference between sexes was not evident at younger ages, some hormonal control might also be involved in the mercury uptake by human hair. The average mercury levels in our hair samples varied among the sampling districts. Tuna is a major carnivorous fish with high mercury accumulations that is often consumed in Japan. The amount of fish consumption and the preference rate for tuna would appear to be responsible for the regional variation in hair mercury levels in Japan.

Recently, a provisional tolerable weekly intake (PTWI) of MeHg was revised by 61st JECFA to 1.6 μg/kg/week, which was about half that of the Japanese standard, and corresponded to a hair level of 2.2 ppm. The distribution of hair mercury levels in Japanese populations in the present study indicated that 25% of the Japanese females of child-bearing age were estimated to be exposed to MeHg over the PTWI level. This would reflect the high Japanese consumption of marine products. However, not only mercury contamination, but also the nutritional benefit may have to be considered when discussing the risk involved in the current level of fish and shellfish consumption in Japan.

Keywords: hair mercury; Japanese population; methylmercury exposure; PTWI; fish

consumption

Methylmercury (MeHg) is formed by saprophyte microorganisms from inorganic mercury compounds in the aquatic environment (ATSDR, 1992). It is accumulated in fish and shellfish through the marine food web. Since the MeHg accumulation increases with the food web, carnivorous fish such as tuna, swordfish and shark often exhibit high levels of mercury. Furthermore, due to the long biological half-life of MeHg, the chemical tends to accumulate throughout the life of fish (Clarkson, 1992). Marine mammals such as whales and dolphins also show high concentrations of mercury. Accordingly, the major route of human exposure to MeHg is the ordinary consumption of fish and shellfish. MeHg is readily absorbed from the gastrointestinal tract and distributed among various tissues including the brain. The permeability of the chemical at the blood-brain barrier is responsible for its hazardousness neurotoxic effect.

A WHO report (1990) concluded that the NOAEL (no observed adversary effect level) for adults is 50 μg/g of the hair mercury level based on the analytical data of MeHg pollution in the past. Since the developing nervous system of the fetus has been considered highly susceptible to the effect of MeHg (Cox et al., 1989), the report also mentioned a possible association with an increased risk to the neurodevelopment of the fetus when maternal hair levels rise above 10 μg/g. Accordingly, recent studies on the health effects of MeHg have focused on the exposure risk to pregnant women and the neuropsychological outcomes in newborns.

In Japan, the provisional regulatory standards of mercury and MeHg in fish and shellfish were determined in 1973 to be 0.4 and 0.3 μg/g, respectively, based on the assumption of a safe intake limit of 0.17 mg mercury/person/week (0.48 μg/kg bw/day). On the other hand, the revised reference dose (RfD) of the US Environmental Protection Agency (EPA, 1997) set the safe exposure limit to MeHg of 0.1 μg mercury/kg bw/day in 1997. This RfD is aimed at the protection of the developing fetus from neurological deficits induced by MeHg in utero, and has been calculated as 1/10 of the benchmark dose obtained in a study of the Iraq incident of 1971-1972. However, since the manner of MeHg exposure in that incident was quite different from the ordinary exposure risk incurred through fish consumption, the Committee on Toxicological Effects of Methylmercury convened by the United States National Research Council (NRC, 2000) reevaluated the RfD. Although the committee scientifically verified the EPA's RfD level, it recommended that its calculation should instead be based on the data obtained in a cohort study conducted in the Faroe Islands (Grandjean et al., 1997). On the other hand, a provisional tolerable weekly intake (PTWI) of MeHg was determined to be 1.6 μg mercury/kg/week at the 61st meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2003). However, a considerable segment of the Japanese population is thought to be exposed to MeHg in excess of the above levels due to their habitually high

consumption of marine products (Yasutake et al., 2003; 2004). Here we reported on a survey of the hair mercury levels in a cross section of representative Japanese sub-populations to estimate the current MeHg exposure levels in Japan.

Current hair mercury levels in Japanese

To estimate the current hair mercury levels in a cross section of Japanese sub-populations hair samples from 12,923 individuals were collected from 2000 to 2004 at beauty parlors, barbershops, and primary schools in 14 districts of 12 prefectures: Hokkaido (Abashiri and Tomakomai Cities), Miyagi, Chiba, Niigata, Saitama, Nagano, Wakayama, Tottori, Hiroshima, Fukuoka, Kumamoto (Kumamoto and Minamata Cities), and Okinawa (Fig. 1).

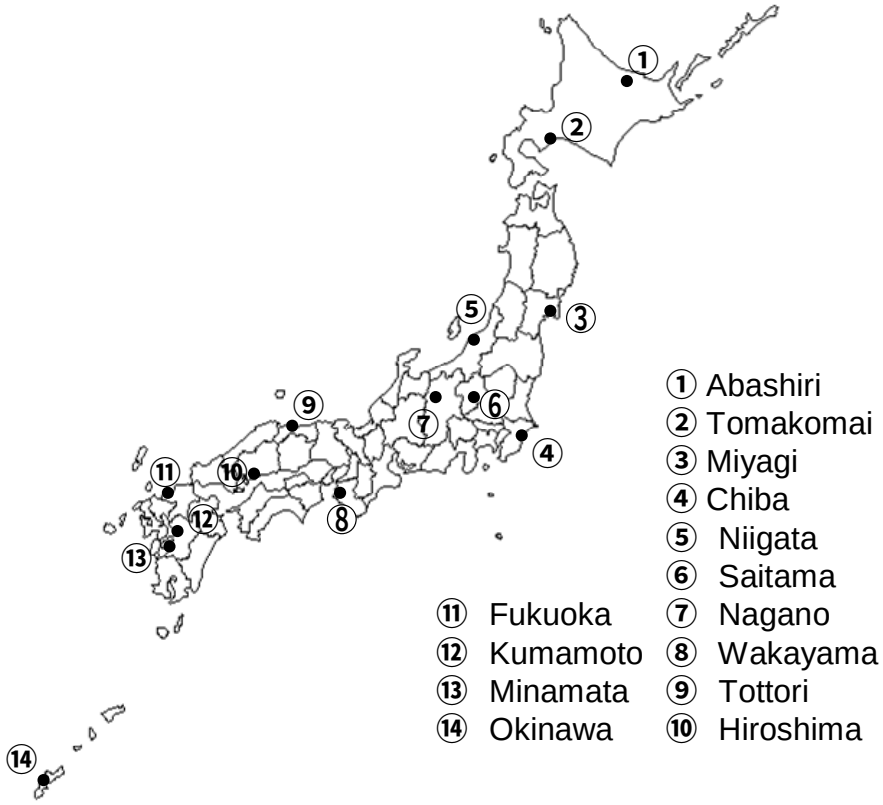


Fig. 1. Hair sampling locations.

Using a questionnaire, we gathered information from each individual on age, sex, amount and species of fish consumed, and artificial waving and coloring of hair. Human exposure to MeHg is mostly accounted for by fish consumption, and thioglycolate used in the lotion for artificial waving effectively removed some of the hair mercury (Yamamoto and Suzuki, 1978; Yasutake, et al., 2003). Total mercury levels of all hair samples thus collected were analyzed by the oxygen combustion-gold amalgamation method using an atomic absorption mercury detector. Since the mercury levels analyzed were distributed in a lognormal manner (Fig. 2A, B), a geometric rather than an arithmetic mean was used as representative of hair mercury levels. Multiple regression analysis revealed that mercury levels were significantly

correlated with several covariates, such as sex, age, the amount of daily intake of total fish/shellfish, a preference for certain fish such as tuna or bonito, and artificial waving (p<0.001). Some detailed results are given below.

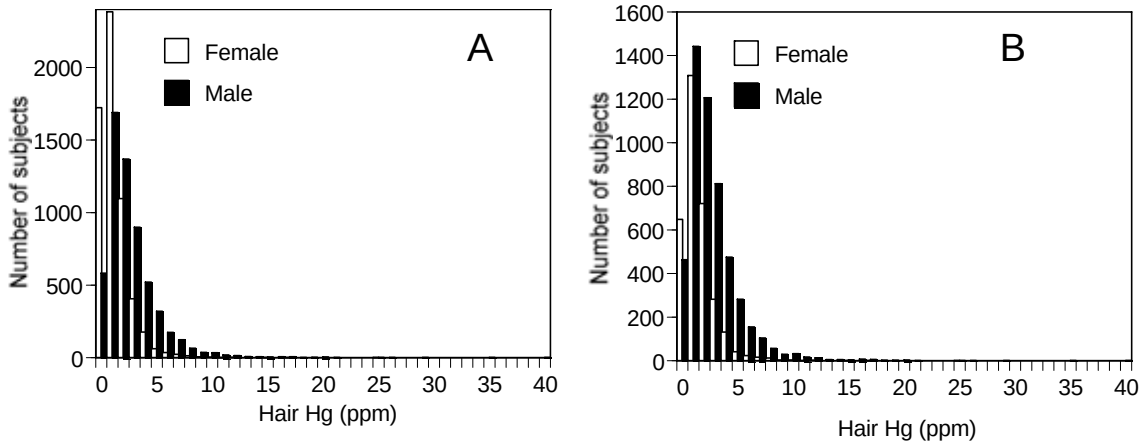


Fig. 2. Distribution of hair mercury content among the total study population (A) and the population without artificial waving (B). Open bar and solid bar indicate female and male populations, respectively.

Sex differences

The mercury levels of 12923 hair samples collected from 14 districts showed a significant sex difference, with females distributed at lower levels than males (Fig. 2A). Geometric means of the levels for males and females in the total population were 2.47 (n = 6,446) and 1.39 μg/g (n = 6,477), respectively. These levels were somewhat higher than those estimated from mercury concentrations in blood or toenails recently reported in western countries (Sanzo, et al., 2001; Guallar, et al., 2002; CDC, 2003). The lower value for females might, at least partly, be due to the high incidence of artificial waving among women. In fact, the frequencies of participants without waving were 52 and 90% for females and males, respectively. Fig. 2B showed the distribution of hair mercury concentrations obtained from the non-waved population. It was evident that female mercury levels were lower than for males. The geometric means for the population without waving were 2.47 and 1.65 μg/g for males (n = 5623) and females (n = 3470), respectively. Even after excluding the contribution from artificial waving, males still showed a higher level than females. Other factors such as the amount of fish consumed might be responsible for the higher male mercury levels. However, the amount of fish consumption shown as per body weight was

found to be equal between male and female (Fig. 3). Since the sex difference in the hair mercury levels became evident after the age of puberty, some hormonal control might be one of possible factors as discussed below.

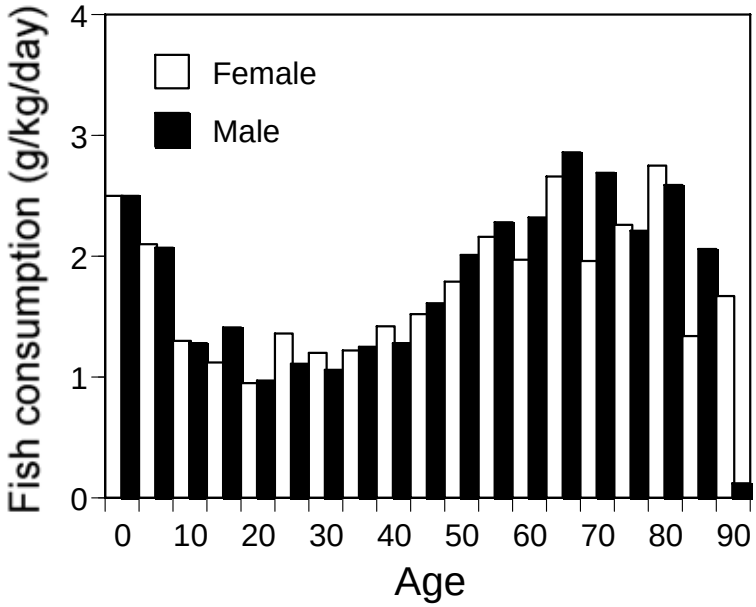


Fig. 3. Age-dependent variation in amount of fish consumption. Open bar and solid bar indicate female and male populations, respectively.

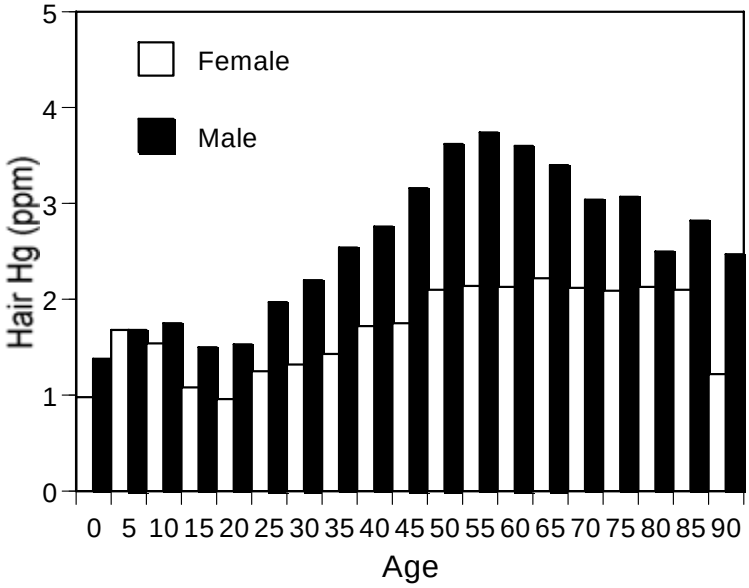


Fig. 4. Age-dependent distribution of the geometric mean of hair mercury content among the population without artificial waving. Open bar and solid bar indicate female and male populations, respectively.

Age-dependent variations

Hair mercury levels varied with age as shown in Fig. 4. Such variations were more

significant in males. Following a transient decline around the 20s, male levels increased into their 50s and 60s, and declined thereafter. The highest levels in the 50s and 60s were mostly twice those in childhood. The age-dependent variation in male hair mercury well fit to fish consumption feature shown in Fig. 3. On the other hand, the age-dependent variations in females were less significant. Although the difference between sexes was not evident at younger ages, the significant increase with age in male mercury levels accounted for a notable sex difference beyond the 20s. Since a marked sex difference subject to modification by hormone treatment has been reported in the tissue uptake and elimination of mercury in MeHg-treated animals (Hirayama et al., 1987), some hormonal control might also be involved in the mercury uptake by human hair.

Regional differences

The average mercury levels in our hair samples varied from 1.72 to 3.81 μg/g for males and from 1.33 to 2.79 μg/g for females among the sampling districts (Fig. 5). Such variations seemed to depend on the total amount of the daily intake of fish/shellfish and on the preference for consuming certain fish. Fish species often consumed in Japan found from the questionnaire in the present study were summarized in Table 2. It should be noted that tuna fish, which has been often consumed by 45% of Japanese, showed extremely high mercury content. Since the mercury content of the second highest fish was less than 1/5 of tuna, tuna consumption supposed to contribute largely to the increase in hair mercury level. Relationships between hair mercury average amounts of the daily intake of fish/shellfish and the rate of preference for tuna consumption. The average consumption of fish/shellfish varied from 50.9 to 115.9 g/day, and the consumption rate of tuna varied from 16.0 to 77.5% (Fig. 6). Tuna is a major carnivorous fish with high mercury accumulations that is often consumed in Japan. The highest rate of tuna consumption was found in Okinawa and Chiba, while Tottori and Minamata had the lowest rate. The highest hair mercury level found in Chiba among all the districts was probably due to the high consumption of tuna there. Although Okinawa also showed a marked tendency to consume tuna, their lower levels of fish/shellfish consumption would tended to depress their hair mercury levels. In contrast, the two districts with the lowest hair mercury levels, Fukuoka and Hiroshima, showed both lower amounts of fish consumption and a lower preference for tuna among all the districts. Thus, the amount of fish consumption and the preference rate for tuna would appear to be responsible for the regional variation in hair mercury levels in Japan.

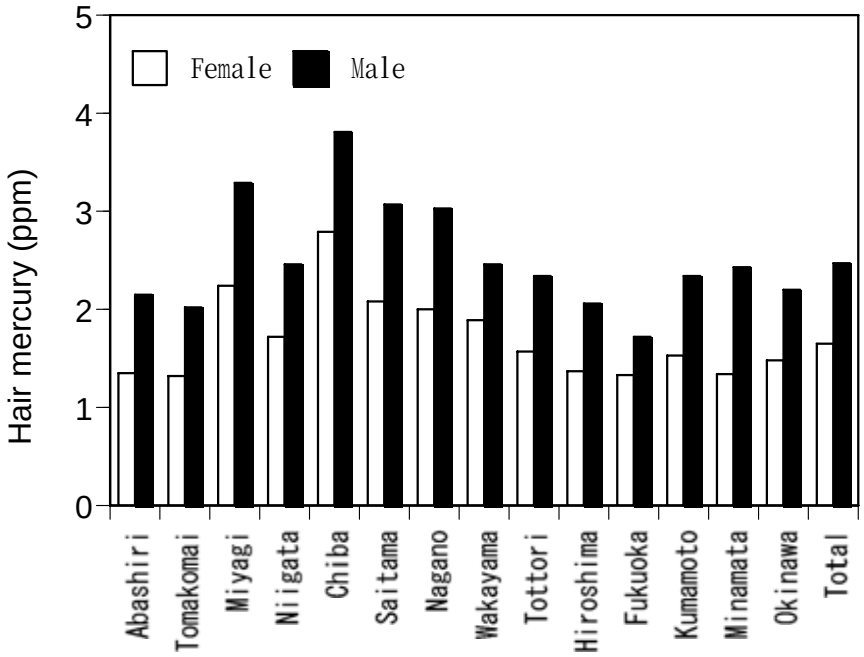
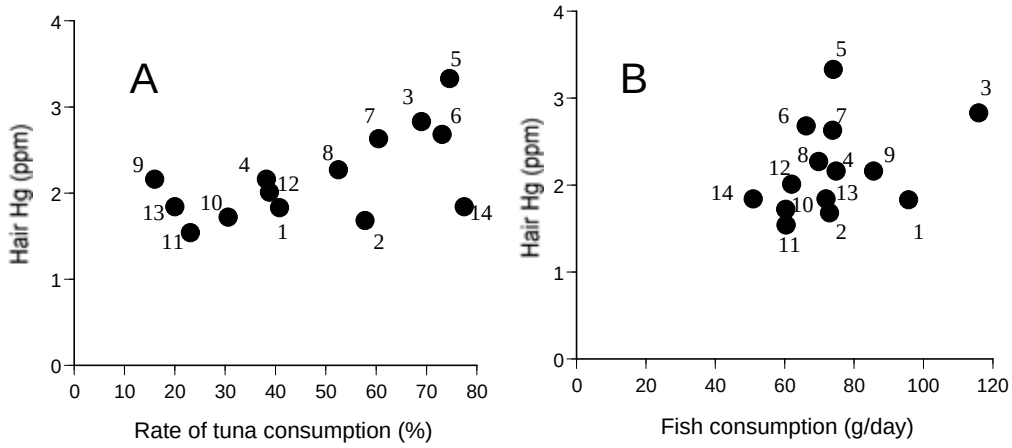


Fig. 5. Geometrical mean of hair mercury levels in 14 districts without artificial waving. Open bar and solid bar indicate female and male populations, respectively.



1: Abashiri; 2: Tomakomai; 3: Miyagi; 4: Niigata; 5: Chiba; 6: Saitama; 7: Nagano; 8: Wakayama
9: Tottori; 10: Hiroshima; 11: Fukuoka; 12: Kumamoto; 13: Minamata; 14: Okinawa

Fig. 5. Relation of hair mercury levels to tuna consumption rates (A) and total amount of fish consumption (B) in 14 districts.

Artificial waving

The lower levels in the adult females might be partly due to artificial hair waving. A frequency of the hair waving above 20's in each district was about five folds higher in females (44.6 to 68.7%) than males (8.4 to 17.1%). Yamamoto and Suzuki (1978) demonstrated that thioglycolate in the artificial waving lotion effectively removed hair mercury. To make sure we treated hair samples from non-artificial hair waved women up to 3 times with waving lotions that were commonly used in Japanese beauty saloons. More than 30% of the hair mercury was removed by a single treatment of the lotions (Fig. 7). Repeated treatments further removed the hair mercury. Removal of a portion of hair mercury was evident also from the longitudinal hair analysis of whole hair samples from females. The typical features for waved and non-waved hairs were shown in Fig. 8. The levels at the root of the artificially waved hair were significantly higher than those at the tip ($p<0.001$) (Table 1). On the other hand, the difference was not significant between the two sides of the non-waved hairs.

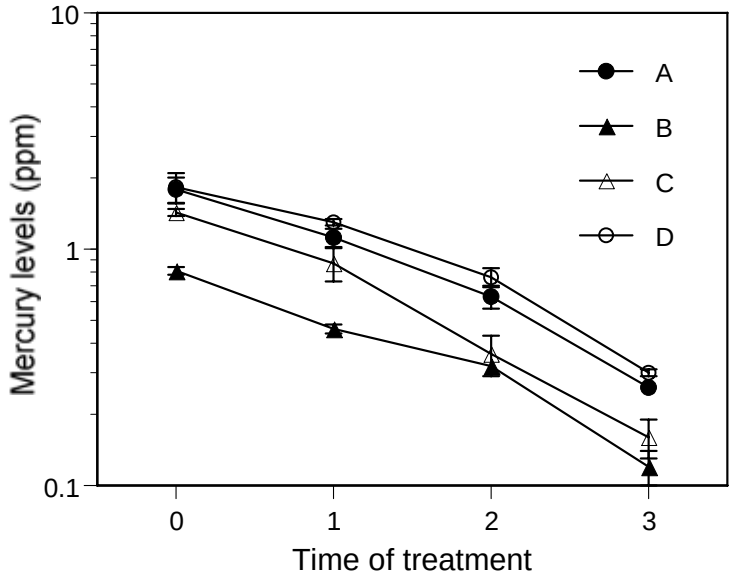


Fig. 7. Effect of artificial waving on hair mercury levels. Non-artificial permanent waved hair samples from 4 women (A to D) were treated with waving lotion up to 3 times. Hair mercury levels were determined after each treatment. Each value represent the mean ± SD of 3 measurements.

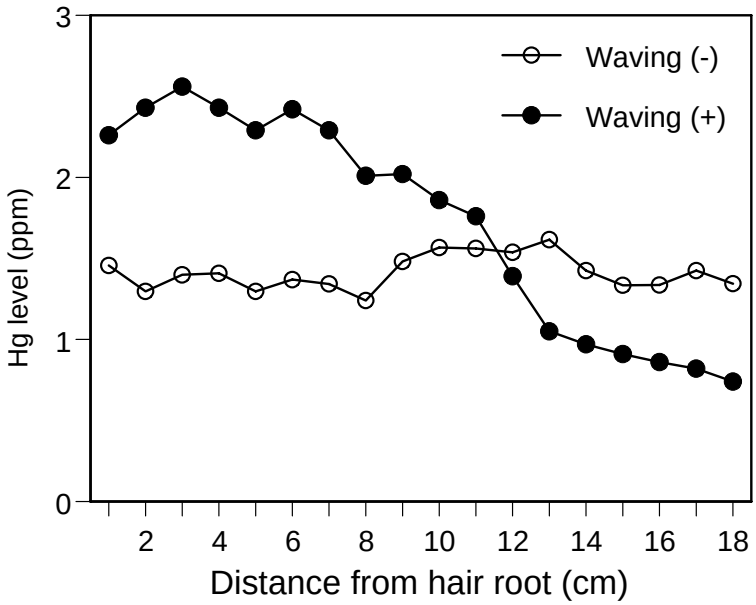


Fig. 8. Whole length analysis of hair mercury levels. Mercury levels of whole hair strands from two women (with and without artificial permanent waving) were analyzed at 1-cm sections.

Table 1. Ratio of hair mercury levels at the hair tip to the root in female Minamata citizens

Artificial waving	No (38)	Yes (98)
Hg Ratio: Tip/Root	0.92 ± 0.21	0.56 ± 0.22

Numbers of hair samples are shown in parentheses

Safe MeHg exposure levels

The exposure level to MeHg can be estimated from hair mercury levels using the following formula (NRC, 2000):

$$d = \frac{C \times b \times V}{A \times f \times bw}$$

where

C = mercury concentration in blood (μg/L) = hair level (μg/g) x 1000/250

b = elimination rate constant (0.014/day)

V = blood volume (9% of body weight)

A = fraction of the dose absorbed (0.95)

f = absorbed fraction distributed to the blood (0.05)

bw = body weight (kg)

d = dose (μg/kg bw/day)

Various levels has been recommended as a safe exposure limit to MeHg in several countries and by international committees. In Japan 0.17 mg mercury/person/week (3.4 μg mercury/kg bw/week) was suggested as a safe exposure limit in 1973. This is almost equal to the former PTWI (3.3 μg mercury/kg bw/week) reaffirmed at the 53rd JECFA meeting (JECFA, 1999), and corresponds to a hair mercury level of about 5 μg/g. On the other hand, 0.1 μg mercury/kg bw/day, which was suggested as an RfD by the EPA (1997) and reevaluated by the NRC (2000), is the lowest level, and corresponds to a hair level of 1.0 μg/g. However, EPA's fish advisories announced that their RfD level had been applied exclusively to recreationally caught fish. In 2003, considering the fetal effect, the 61st JECFA has revised the PTWI to 1.6 μg mercury/kg bw/week, based on the results of Faroes and Seychelles studies. The new PTWI corresponds to a hair mercury level of 2.2 μg/g. Recently, Japanese Food Safety Commission (2005) recommended for pregnant women to reduce the previous PTWI by 60% to 2.0 μg mercury/kg bw/week, corresponding to a hair mercury level of 2.75 μg/g.

Table 2 Frequencies (%) of sub-populations exceeding certain levels in current Japanese

Hair mercury (μg/g)	0≤	**1<**	2<	**2.2<**	**2.75<**	**5<**	10<
Male (total)	100	90.1	60.9	55.7	42.9	14.5	2.1
Female (total)	100	70.4	29.8	24.8	15.8	2.4	0.2
15-49 years female							
(total)	100	63.4	22.2	18.9	10.7	1.3	0.2
(without waving)	100	**73.7**	29.2	**24.9**	**14.7**	**1.7**	0.1
Total	100	80.2	45.1	40.0	29.3	8.4	1.1

The cumulative frequency of hair mercury levels in our survey was shown in Table 2. The districts that exceeded the 5 μg/g which was recommended in Japan and by the former PTWI (JECFA, 1999) were less than 10% of the total population surveyed. When restricted to females of child-bearing age, 1.7% of the sub-population had hair mercury concentrations exceeding that level. However, the majority (87% of the total, 80% of females, 74% of females child-bearing age from 15 to 49 years, and 91% of males) exceeded 1 μg/g. On the other hand, the average hair mercury levels of all Japanese females (1.65 μg/g, without waving) and females of child-bearing age (1.43 μg/g, without waving) were lower than the new PTWI level for pregnant women (JECFA, 2003). However, considerable population segments (31% of all females and 25% of females of child-bearing age) exceeded the PTWI level, which was determined using NOEL/BMD for maternal hair mercury levels reported in the Faroes (12 μg/g) and Seychelles (15.3 μg/g) with an uncertainty factor of 6.4 (JECFA, 2003). Although it is difficult to assess the risk level for females of child-bearing age, they may not be urgently at risk, since none of them exceeded the NOEL/BMD levels obtained in

the Faroes and Seychelles.

For pregnant women and those who may become pregnant, The Ministry of Health, Labor and Welfare, Japan (2003) recently announced a program to regulate the consumption of several kinds of fishes and whales that showed high concentrations of mercury. Such a program may be sufficiently effective to bring about some reduction in fish consumption in Japan. However, not only the risk of mercury contamination, but also food habits and nutritional benefits may have to be considered when determining a regulatory standard of fish and shellfish. Sufficient and accurate information must be provided to reach an appropriate decision on fish consumption. Hair analysis may, at least in part, contribute to such decisions by providing information on the MeHg exposure levels of each individual.

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[WS-5]

CADMIUM EXPOSURE OF PRESCHOOL CHILDREN IN GENERAL POPULATIONS IN JAPAN

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[Objectives] The Japanese people are known to have high environmental exposure to cadmium. The present study is initiated to estimate the current levels of cadmium exposure of children in general populations in Japan.

[Methods] The investigation was carried out in Miyagi Prefecture. Food duplicate samples and spot urine samples of 260 children of the age of 4 or 6 were collected and analyzed by ICP-MS after the pretreatment of wet digestion. Urinary α1-MG, β2-MG and NAG were also measured as markers of renal tubular dysfunction after environmental exposure to cadmium. The nutritional intakes of energy, protein, and many other nutrients were concluded with reference to the 5th Japanese standard food table.

[Results] The dietary intake of cadmium was found to be 11.7 μg/day/capita in total and to change from 7.6 μg/day/capita to 16.0 μg/day/capita by kindergarten group depending on the regions. The average daily intake of boiled rice in total was 207.4 ± 83.4 (M ± SD) for the group and it can be estimated that boiled rice contains 3.5 μg/day/capita (GM). This figure indicates that 43.9 % of total daily cadmium come from boiled rice. Correlation of coefficient between the dietary intake of cadmium and the boiled rice intake of cadmium was 0.783 ($p<0.01$) in individual of 260 and 0.613 ($p<0.01$) in kindergarten group of 13. Significant correlation was found between the urinary cadmium excretion and urinary excretion of N-acetyl-β-D-Glucosaminidase (NAG).

The geometric mean of cadmium in urine turned out to be 0.96 μg/L in total. Correlation of coefficient between the dietary intake of cadmium and the urinary excretion was 0.357($p<0.01$). Significant correlation was found between the urinary cadmium excretion and urinary excretion of NAG.

[Conclusions] Further analyses must be necessary to investigate the relationship between cadmium intake and urinary excretion on one hand and food composition on the other hand, and the difference of cadmium intake and excretion between adults and children.

[WS-6]

CONTAMINATION BY CADMIUM AND MERCURY OF THE WATER, SEDIMENT AND BIOLOGICAL COMPONENT OF HYDROSYSTEMS AROUND HANOI

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Industrial and municipal waste water is directly discharged into river of Hanoi, Vietnam. Water and sediment and biological component were collected from different sites of two rivers in Hanoi city and examined for total heavy metals contents. Concentration of cadmium(Cd) and mercury(Hg) ranged from 0.06-1.49, 0.09-3.34μg/l in river water, ranged from 0.80-1.79, 0.078-1.136 ppm in river sediment, ranged from 0.53-24.94, 0.036-1.567 ppm respectively. the accumulation of Cd and Hg in water hyacinth increased with the increase of Cd and Hg concentrations in water and SPM; and the capacity of accumulation is in order: leaves< stems<roots in water hyacinth. The concentration of Cd and Hg in water and sediment samples increases along Nhue River from upstream to downstream. After confluence with Tolich River, the concentration of Cd and Hg was highly increased.

Introduction

Hanoi is the capital of Vietnam and located in the Red River Delta in Northern Vietnam. After the shift of Vietnam's economy from a central planned economy to a market oriented economy in 1986, the population density of Hanoi has rapidly increased. Hanoi has about 2.25 million residents (estimation in 2002 by Hanoi Statistical Office) and its population increases at an annual rate of more than 2%. There are more than 90 factories in the city, consuming nearly 148,000 tons of coal and 12,000 tons of petroleum per year (Nguyen, 1997). Most of the factories were built after 1954 in the periods of 1950 – 1960 and 1970 – 1980.

In the Red River Delta area, farmers have a long history of using river water to irrigate and applying sediment as an organic amendment to keep soil fertile. Recently, however, it has been reported that application of sediment or sludge to agricultural soil might result in accumulation of heavy metals in soil (van den Berg, 1993; Hooda and Alloway, 1996). Of the heavy metals, cadmium, and mercury are potential pollutants. The objective of this research is to evaluate heavy metals pollution in the water and sediment from the river system in Hanoi in order to understand the risky effects of heavy metals to agricultural products. In this

research, water, sediment and water hyacynth samples were collected from six sites in Nhue and Tolich rivers and determined for total concentration of heavy metals.

Materials and Methods

Samples and Sampling Sites

River system of the studied area and sampling sites are illustrated in Figure 1, and the location of sampling site is shown in Table 1.

The Nhue River connects with the Red River at Thuy Phuong Dam and it runs mostly outside of Hanoi. The Tolich River begins at the West Lake and receives 22,000 m^3 per day of industrial waste water from 33 factories and 29,000 m^3 per day of municipal waste water (Nguyen, 1997).

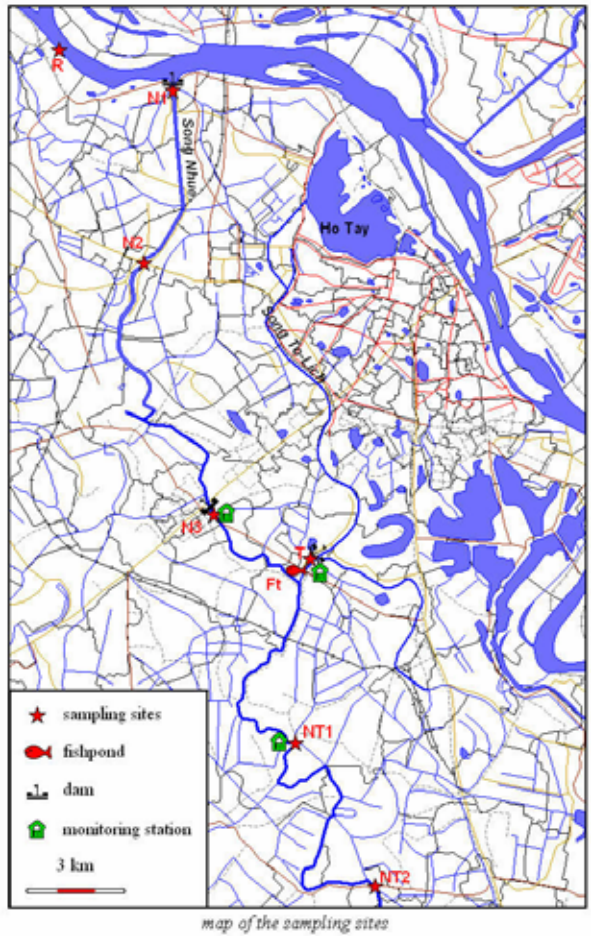

Figure 1. River system and sampling sites in Hanoi city

The depth of river water at the time of sampling was about 1-3m. Water samples (50 ml) were collected and acidified with concentrated HNO_3. Sediment samples (500 mg) were collected by Shipek drag sampler at each site (20 cm from the surface). Water and sediment samples were then stored under frozen condition.

Table1. Location of sampling sites

River	Sample	Location
Nhue	N1	Thuyphuong dam, Tuliem, Hanoi
	N2	Caudien, Tuliem,Hanoi
	N3	Cauden, Hadong
	NT1	Khetang, Hatay
	NT2	Cauchiec, Hatay
Tolich	T	ThanhLiet dam, Thanhtri, Hanoi

Determination of Cd and Total Hg (T-Hg) in water.

Determination of Cd: The methods for analysis of heavy metals are described elsewhere (Neal et al., 1996, 1997; Jarvie et al., 2000), and only a brief summary is provided here.
Heavy metals analysis in this study was undertaken for dissolved fraction. The dissolved fraction was measured by filtering samples and acidifying filtrates on the day of sampling. Samples were acidified to 1% v/v concentrated super pure nitric acid, to ensure that precipitation and adsorption of heavy metals did not occur to a significant degree during storage prior to chemical analysis. For the campaign- sampling, samples were filtered using 0.45μm Whatman cellulose nitrate sterile membranes (47 mm diameter) to ensure consistency with the data collected by the Environment Agency (EA).
Water samples were analyzed by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). During the initial analyses, contamination tests were run by filtration and storage of blank samples. Quality control standards were included in the routine analyses and these standards were checked against an international quality standard prepared by (National Research Council Canada. River water Reference Material for Trace Metals – SLRS-4)

Determination of Hg: Total mercury in water was determined by cold vapor atomic absorption spectrometry (CV-AAS) according to the method of Akagy and Nishimura (1991). The method involves sample pre-concentration by extraction with dithizon in Toluene and digestion of samples with HNO_3, $HClO_4$ and H_2SO_4; followed by reduction to Hg^0 by $SnCl_2$. The detection limit was 0.1 (ppb).

Determination of heavy metal in sediment and biological components.

Determination of Cd: Thirty mg of sediment and biological samples was digested in closed Teflon reactor (Savillex) with the HF-HNO_3-HCl acid treatment to determine total concentration of heavy metals. Two ml HF concentrated , 250μl HNO_3 concentrated and 750μl HCl concentrated were added to the samples, heated for 2h at 110^{o}C, and then heated without cover until dryness at 110^{o}C. After evaporation to dryness, the residues were

completely re-dissolved in 150µl HNO_3 concentrated on heating plate and after cooling make up 10 ml with Millie-Q water. The solution was analyzed for heavy metals by ICP-MS. Determination were made in duplicated and the relative deviation of duplicate values was less than 5%. Quality control standards were included in the routine analyses and these standards were checked against an international quality standard prepared by (Community Bureau of reference- Commission of the European Communities. Certified Reference material – CRM 320).

Determination of Total mercury: Total mercury in sediment and biological samples was determined by cold vapor atomic absorption spectrometry (CV-AAS) according to the method of Akagy and Nishimura (1991). The method involves digestion with HNO_3, $HClO_4$ and H_2SO_4 followed by reduction to Hg^0 by $SnCl_2$. The detection limit was 0.1ng/g. Accuracy was ensured using certified reference material (DORM-2 dogfish muscle prepared by the National Research Council Canada and NIES -12 Marine sediment prepared by National Institute for Environment Studies, Japan). The total analytical precision of this analysis was estimated to be 3.9 %.

Result and Discussion

Heavy metals in water and suspended particulate matters

Total concentration of Cd and Hg in river water is shown in Table.2. It varied considerably among the samples. The concentration of cadmium(Cd) and mercury (Hg) ranged from 0.045 - 2.141 and 0.082 - 3.118 (µg/l), respectively. The anthropogenic emission sources of heavy metals in the Hanoi city are atmosphere emission, agricultural activities, domestic and industrial waste. In order to assess the possible anthropogenic influences on the enhancement of heavy metals contents**.** Significant anthropogenic influences on heavy metals distribution have been seem in the Tolich river with high concentration of Cd and Hg.

Cadmium and mercury in Tolich River show higher concentrations than in Nhue River. The increase of Cd and Hg caused by the deposition of dust emitted and waste from various industries.

Table 2. Concentration of heavy metals in river water

Parameter (Unit)	Samples					
	N1	N2	N3	NT1	NT2	T
Cd (µg/l)	0.06±0.02	0.07±0.03	0.07±0.02	0.31±0.03	0.32±0.05	1.49±0.07
Hg (µg/l)	0.09±0.03	0.11±0.03	0.11±0.04	1.98±0.09	1.13±0.07	3.34±0.08

Heavy metals in sediment and suspended particulate matter

The concentration of heavy metals in sediment of Nhue and Tolich River was shown in Table 3. As the water, concentration of heavy metals was increased along Nhue River. The increase of Hg and Cd contents is very clear. High concentration of Hg and Cd in SPM showed that Cd and Hg interacted with particulate matter via physical and chemical adsorption so risk of higher bioavailability if the oxygen increases in the water column.

Table 3. Concentration of Cd and Hg in river Sediment

Parameter (Unit)	Samples					
	N1	N2	N3	NT1	NT2	T
Cd (ppm)	0.80±0.32	1.05±0.13	0.63±0.11	1.89±0.25	1.62±0.16	2.79±0.39
Hg (ppm)	0.078±0.011	0.078±0.013	0.132±0.015	0.237±0.019	0.233±0.023	1.136±0.076

Table 4. Concentration of Cd and Hg in SPM

Parameter (Unit)	Samples					
	N1	N2	N3	NT1	NT2	T
Cd (ppm)	0.53±0.18	0.59±0.21	0.96±0.13	4.52±0.12	3.82±0.11	24.94±1.19
Hg (ppm)	0.036±0.023	0.034±0.021	0.065±0.013	0.231±0.024	0.139±0.017	1.567±0.165

Heavy metals in water hyacinth.

Water hyacinth was collected from 5 different sites (N2, N3, T, NT1, NT2). Concentration of Cd and Hg in the different parts of water hyacinth such as roots, stems and leaves was determined. The results are shown in table 5.

Table 5. Concentration of Cd in different parts of water hyacinth (Eichhornia Crassipes)

Sampling point	Cd(ppm)		
	Leaves	Roots	Stems
N2	0.112	0.812	0.202
N3	0.108	0.798	0.231
T	0.687	22.343	7.361
NT1	0.234	2.997	0.567
NT2	0.323	1.876	0.396

Table 6. Concentration of Hg in different part of water hyacinth (Eichhornia Crassipes)

Sampling point	Hg (ppm)		
	Leaves	Roots	Stems
N2	0.235	0.421	0.339
N3	0.234	0.398	0.323
T	1.234	2.837	1.134
NT1	0.545	0.798	0.723
NT2	0.562	0.812	0.698

The results indicated that water hyacinth accumulated the highest concentration of metals in roots (22.343 ppm for Cd and 2.837 ppm for Hg at Tolich river). Only relatively small amount of Hg (1.134 ppm at Tolich river) was translocated from roots to the stems and leaves, while Cd was translocated at much higher concentration (7.361ppm at Tolich river). This demonstrated that Cd was much more mobile than Hg. The high accumulation of Cd and Hg in the roots and stems of water hyacinth suggested that water hyacinth can be used as biological marker in monitoring heavy metals pollution. The results also showed that the accumulation of Cd and Hg in water hyacinth increased with the increase of Cd and Hg concentrations in water and SPM; and the capacity of accumulation is in order: leaves< stems<roots in water hyacinth. This observation was in consistence with most studies which also reported the higher concentration of metals in roots than in stems and leaves.

Conclusion

Water and sediment from Nhue and Tolich rivers running through the industrial and densely populated of Hanoi city are polluted with heavy metals to various degrees. The water pollution is observed in Tolich River and after confluence point with Tolich in Nhue River.
Heavy metals were accumulated and deposited in sediment and water hyacinth. Risk of higher bioavailability if the oxygen increases in water column. Cadmium, Hg are all possible pollutant in the area. All of them are most hazardous to crops and impacted potentially toxic on environment.

Acknowledgements

The authors were grateful for financial assistance from National Institute for Minamata Disease and French-Vietnamese water quality program.

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[WS-7]

GENETIC ENGINEERING OF *ESCHERICHIA COLI* FOR BIOREMEDIATION OF MERCURIAL CONTAMINATED-ENVIRONMENTS AND DETECTION OF MERCURY IN ENVIRONMENTS

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Mercury is one of the most important environmental pollutants as a global level that has been released into environment in substantial quantities by natural events and anthropogenic activities. The effect of this pollution on the ecosystem and human health are growing concerns. To prevent the environmental mercury poisoning incidents, technologies for treating mercury-polluted environments, and measuring and monitoring of mercury in the environment are increasingly urgently required.

In the present study, a new *mer-ppk* fusion plasmid, designated pMKB18 was first constructed for mercury remediation by using a fusion of the well-understood *mer* operon (*merR-o/p-merT-merP-merB*) from *Pseudomonas* K-62 with a bacterial polyphosphate kinase gene, *ppk*, determining polyphosphate kinase enzyme, a key enzyme for polyphosphate synthesis, from *Klebsiella aerogenes*, and then its feasibility in mercury remediation was evaluated (Fig. 1).

The alginate-immobilized cells engineered to express mercury transport system, organomercurial lyase and polyphosphate efficiently removed organic and inorganic mercury from contaminated wastewater over a wide concentration range of mercurials, probably via intracellular accumulation of mercury mediated by *ppk*-specified polyphosphate. During the incubation period, the accumulation rates were around 891 and 776 nmol Hg/mg cells (dry weight) from Hg^{2+} and $C_6H_5Hg^{+}$-contaminated wastewater, respectively. Bioaccumulation of mercury was not inhibited by environmental stress such as a synthetic detergent, chlorine and several heavy metals. In addition, the immobilized cells are also available for remediation of Cd^{2+}, Pb^{2+} and Cr^{6+}. The immobilized cells could be used repeatedly at least three times without large loss of the activity of mercury removal (Fig. 2).

From these results, the polyP-mediated mercury accumulation from wastewater described here could serve as a useful strategy not only for simultaneous removal of

organic and inorganic mercury but also for removal of the other heavy metals from contaminated wastewater.

Next, a new whole-cell bacterial sensor for the detection of low concentration of mercury in environment was constructed by gene fusion between a *mer* operon (*merR-o/p-merT*) from *Pseudomonas* K-62 and a promoterless *luxAB* gene from *Vibrio harveyi*. The luminescence-based biosensor was evaluated for the selectivity and sensitivity of the detection of mercury. Cadmium, lead, chromium and zinc ions did not interfere with the assay even at same concentration compared to Hg^{2+}. Methylmercury, phenylmercury and mercuric sulfide also did not affect the biosensor. These results reveal that the specificity of the construct is restricted to bioavailable Hg^{2+}. The sensitivity of the biosensor was improved by decreasing the cell density in the bioassay in addition to genetically expressing an Hg^{2+} transport system which was expected to increase the amount of *mer* operon-inducing mercury in the cytoplasm. In optimized assay conditions, the lowest detectable concentration of Hg^{2+} was 2 pM with 1 ml sample (Fig. 3). This detection limit is enough to detect this compound in many contaminated and some pristine environmental samples. Rapid and sensitive measurements of mercury compounds are urgently required in various fields such as environment, food industry and medicine. The assay by using the biosensor developed in this study is simple, sensitive, gives results in a relatively short time and would allow the assessment of the biologically available fraction of Hg^{2+} in many contaminated and in some pristine environments.

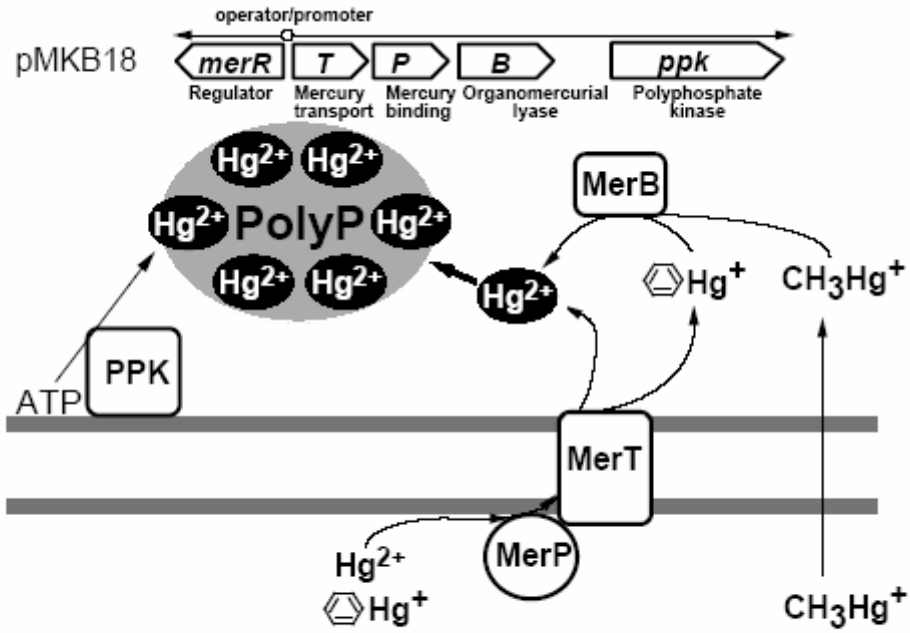

Fig. 1 Schematic diagram of a recombinant plasmid for remediation of mercurials

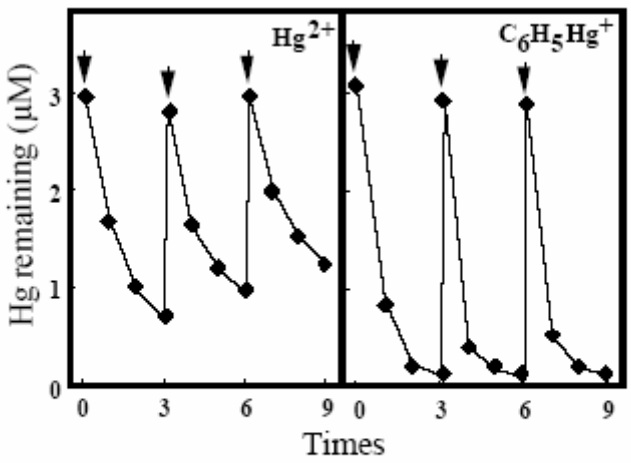

Fig. 2 Removal of mercury from wastes by immobilized biobeads

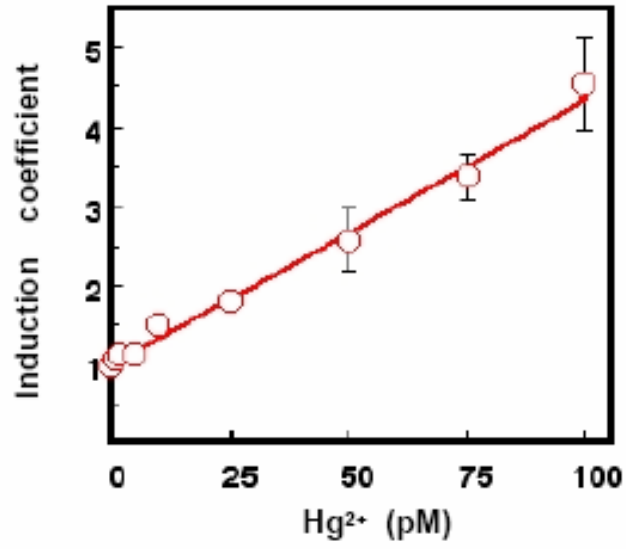

Fig. 3 Callibration curve for detection of Hg^{2+} by *mer-lux* biosensor

[WS-8]

EVALUATION OF THE EFFECTS OF LOW-LEVEL CADMIUM INTAKE ON RENAL FUNCTION AND BONE METABOLISM OF MOTHER RATS

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Abstract

To evaluate the effect of low-level cadmium (Cd) intake, the renal function and bone metabolism in mother rats was investigated together with the effect of Cd intake and pregnant-lactation.

Female Wistar rats (6 weeks old) were given Cd (as $CdCl_2$) at a dose of 1, 2, and 5 mg Cd/kg/day by gastric tube daily for 6 consecutive days a week for 10 weeks. From 3 weeks after oral Cd administration, female rats were paired with male rats at 1:1 ratio for 1 week. Cd was given continuously during the pairing, pregnancy and lactation. After delivering, mother rats lactated to newborn rats of 4 male and female each.

Intestinal absorption rate of Cd was estimated about around 0.5%. Cd intake in consideration of intestinal absorption rate was estimated with 2, 7, and 14 μg/kg/day respectively among the experimental groups of 1 mg/kg/day, 2 mg/kg/day, and 5 mg Cd/kg/day. Cd contained in diet was around 0.1% to the amount of orally administrated Cd. In the case of oral Cd administration, Cd, as the chemical form of Cd-MT, is bound by metallothionein (MT) in intestinal tissue, and more Cd was accumulated in kidney rather than in liver. The increased urinary excretion of β2-microglobulin (β2-MG) and N-acetyl-D-glucosaminidase (NAG) was found both in the groups of 2 mg Cd/kg and of 5 mg Cd/kg, in the group of lactation load. But in the group of Cd intake without lactation, this increase of urinary excretion of β2- MG was not found in both these Cd intake groups 2 and 5 mg Cd/kg. Without the renal dysfunction, the increase of osteoid volume of femur of mother rats resulted by lactation. On the other hand, the effect of Cd intake together with lactation caused the significant decrease of bone mineral density and of bone bending strength in each of groups of 5mg/kg and 2mg/kg. Additionally, the increase of the femoral osteoid volume and of the urinary excretion of calcium also resulted in both groups. Urinary excretion of deoxypyridinoline (DPyr) as the biomarker of bone absorption increased by lactation of all experimental groups compared to that of control group. Although the urinary excretion of

DPyr increased, the remarkable increase of osteocalcin of the index of bone formation was not found. The decrease of bone mineral density, bone bending strength and bone mineral content were found prominently. Although the effect of lactation load to bone metabolism was remarkably than that of Cd intake of mother rats, the additive adverse effect of Cd intake was also recognized.

It was suggested that effect of Cd intake to mother bone metabolism must be evaluated considering gestation and lactation

Introduction

Cadmium (Cd) is a ubiquitous heavy metal that is mainly taken in by the body through the diet, smoking, and environmental exposure. Cd is a toxic metal that accumulates selectively in liver and kidney. Cd is detoxified and stored mainly in these two organs as metallothionein (MT)-bound Cd(Cd-MT). Essential elements, such as calcium (Ca), iron, zinc, copper, and vitamin-D interferes each other on an intestinal absorption and/or toxicity of Cd (Friberg, 1974; Tuchiya, 1978; Flanagan et al., 1978; Hamilton and Valberg, 1974; WHO IPCS, 1992; Ohta et al., 1995; 2002). Cd metabolism is similar in man to that observed in rodents and other mammalian species. Cd compounds are poorly absorbed orally. But once absorbed, Cd is very efficiently accumulated in the body, particularly in liver and kidney, and only trace amount is excreted in urine and feces. When the renal Cd concentration saturated to the toxic level causing renal proximal tubular dysfunction, an increased urinary excretion of β2-microglobulin(β2-MG), N-acetyl-D-glucosaminidase (NAG), amino acid, glucose, and Cd are manifested. The chemical form of Cd is an important factor for the distribution and for the toxicity of absorbed Cd in the body (Ohta et al., 2000; WHO IPCS, 1992). MT bind Cd (MT-Cd) and eliminates the toxicity of Cd (Cherian et al., 1976; Nomiyama and Nomiyama, 1982, 1986; ILZRO, 1989; Chan et al., 1993; Dorian et al., 1995). However, it has been also well known that when MT-Cd leaked into blood circulation, the toxicity of this external MT-Cd to kidney is more toxic compared to that of inorganic chemical form of Cd (Nomiyama and Nomiyama, 1982, 1986; ILZRO, 1989).

Itai-itai disease is well known as the disease of multi-delivery old mothers caused by chronic Cd toxicity. Renal proximal tubular dysfunction reflected by an increased urinary excretion of proteinuria, glucoseuria, and enzymeuria were well known, and the value of 200 ug/g has been urged for the critical Cd concentration. Also the disorder of bone metabolism was observed symbolically as well as renal dysfunction (WHO IPCS, 1992; Friberg, 1974; Tuchiya, 1978). The characteristic toxic effect observed after repeated oral exposure of animals to Cd is nephrotoxicity, usually limited to the proximal tubules.

Recently, it has been reported by Ohta et al. (2000b) that the critical Cd concentration in kidney was varied depending to the condition of Cd exposure such as the duration and dosage of Cd. Namely, the value of critical concentration was able to be changed when the urinary excretion of NAG, glutathione S-transferase, and amino acid was used as the biochemical

indexes. Moreover, the manifestation of bone metabolism disorder was observed before and after that of renal dysfunction. The disorder of bone metabolism was not secondary toxic effect to the renal dysfunction by Cd toxicity, and it was depended to the case by case of Cd exposure condition (Ohta et al., 2000, 2002b). So far, a number of oral administration of Cd have been carried out. However, a little study have been carried out using mother animals administered orally Cd and lactation burden to mother rats to evaluate the relationship between the renal dysfunction and bone metabolism disorder caused by Cd intake.

The present study was investigated to evaluate the effect of low level Cd intake in mother rats considering the effects of pregnancy and lactation under normal condition without surgical treatment.

Materials and Methods

Animals;

Female and male Wistar strain rats (6 weeks old) were used in this experiment after pre-breeding for one week of 5 weeks old rats. The animals were fed a standard rodent diet (CE-2 diet Nippon CLEA) and water ad libitum, and were kept under pathogen-free conditions at a room temperature of 23±1degree Celsius on an alternating 12-hr light/cark cycle. Female rats were given Cd (CdCl2) at the dose of 1, 2, 5 mg Cd/kg/day by gastric tube for 6 conservative days a week. At the third week, rats was paired with male rats and kept for one week. Pregnancy was confirmed by checking vaginal plug and weighing body weight. After delivery newborn rats, a part of mother rats lactate for 4 weeks, and the other mothers did not have lactation. Control rats were given distilled water and experimental control rats were given Cd without pairing with male rats. After the lactation for 4 weeks and collected 24 hr excreted urine under 4 degree Celsius, animals were sacrificed by collecting blood from heart and circulated with physiological saline to washout remaining blood in organs. Kidney, liver, femur, and another organs were collected immediately.

Biochemical examination;

Urinary sample was used for the determination of biochemical indexes of β2-microglobulin(β2-MG), N-acetyl-D-glucosaminidase (NAG: NAG test Shionogi Inc.), amino acid (AA), glutathione S-transferase (GST), protein, deoxypyridinoline (DPyr), pyridinoline (Pyr), creatinine, and etc. Plasma sample was used for the determination of BUN, GOT, GPT, ALP, intact-Osteocalcin (GBP: RIA from Biomedical Technologies Inc.).

Analysis of Bone metabolism indices;

Bone mineral density was analyzed using the NIH Image analysis program to the radiographic density of femur bone with the microdensitometer (DM) method after the photography by Softex x-ray equipment with the standard aluminum scale.(Inoue et al., 1983;

Sanchez et al., 1981; Ammann et al., 1992). Bone bending strength of femur was determined by 3 point method. Osteoid volume (%) was determined and calculated after the Yoshiki staining of osteoid in bone tissue of femur (Yoshiki, 1973).

Metal analysis;

Elements included Cd, zinc, copper, calcium in organs and femur were determined by flame or flameless atomic absorption spectrometry (Hitachi Zeeman 180) after tissue digestion with nitric acid in microwave.

Statistics;

Statistical analysis was carried out by ANOVA with the tests of PLSD and Scheffe at the significant level of $p<0.05$.

Results

In this experiment, to estimate the dose of oral Cd administration, we referred to the result of clinical epidemiological study for Cd concentration in kidney of the Japanese who did not recognize disease (by Yoshida et al., 1998). In addition, we also referred to the experimental study of Cd accumulation of long-term Cd oral administration (Ohta et al., 1998, 2000, 2002a). And we estimated that oral administration group of 1mg Cd/kg in this experiment was the similar level to daily Cd intake level in human.

Cd accumulation in liver and kidney was dependent on an increase of oral Cd administration. The difference in Cd accumulation by gestation and lactation was not recognized in both organs. Much more Cd was accumulated in kidney rather than in liver. (Fig.1) In case of Cd injection experiment, it has been reported that Cd is accumulated in liver rather than in kidney.

The increased urinary B2-MG excretion was found in the lactation group of both groups of 2 mg Cd/kg and 5 mg Cd/kg. (Fig.2) In the 5 mg Cd/kg group, the significant increase of urinary excretion of β2-MG and NAG, reflecting renal dysfunction, was observed especially in mother rats lactating newborn for 4 weeks. On the other hand, in the case of Cd intake without lactation, this increase of urinary excretion of B2-MG was not found in both these Cd intake groups. Namely, in the case of Cd intake of about 2- or 5-folds of daily Cd intake of human in this experiment, it was found that renal dysfunction was prominently caused by Cd intake together with lactation. The meaningful change for urinary excretion of amino acid and protein was not recognized.

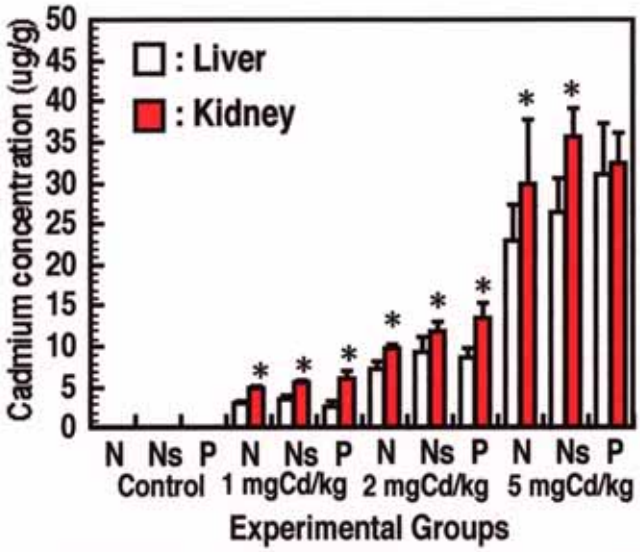


Fig. 1 Cadmium concentration in liver and kidney.

N: Non pregnancy Ns: Non Suckling
P: Pregnancy and Suckling
★:$p < 0.05$

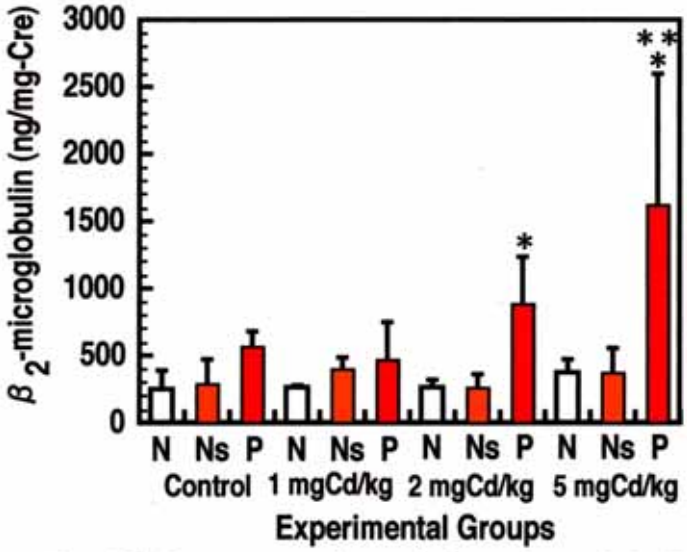


Fig. 2 Urinary excretion of β_2-microglobulin.

N: Non pregnancy Ns: Pregnancy and Non Suckling
P: Pregnancy and Suckling
*: $p < 0.05$ to N of Control.
**: $p < 0.05$ to P of Control.

As similar with the case of Cd accumulation in liver and kidney, the Cd accumulation in the femur was increased corresponding to the increased amount of Cd intake (Fig.3).

Depending on the increased Cd accumulation in femur, bone mineral density of the femur decreased markedly in the lactation group (Fig.4). However, even if Cd intake was the same and the Cd accumulation in femur was the same, the significant decrease of bone mineral density of femur was not found in the Cd intake group of non-lactation. Although the

decreased bone mineral density of femur was observed in the control group, the significant decrease of bone mineral density of femur was found in both the groups of 2 and 5 mg Cd/kg. On the other hand, the bone maximum bending strength of femur was measured by three points method. Bone maximum bending strength decreased according to the increase of Cd accumulation in femur as well as the case of bone mineral density. The significant decrease of bone maximum bending strength was found in all experimental groups of lactation load. Moreover, in both the groups of 2mg Cd/kg and 5mg Cd/kg, the bone maximum bending strength was decreased significantly compared with the decrease caused by lactation of 1 mg Cd/kg group and control group (Fig.5).

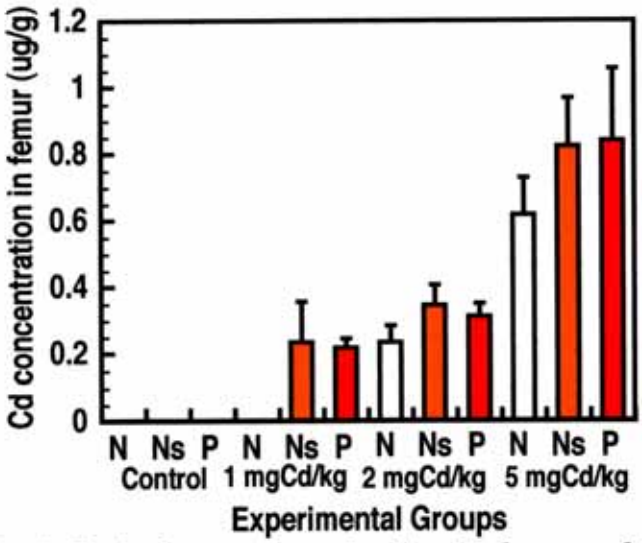


Fig. 3 Cadmium concentration in femur of female rats after oral cadmium administration and pregnancy.

N: Non Pregnancy Ns: Pregnancy and Non Suckling
P: Pregnancy and Suckling

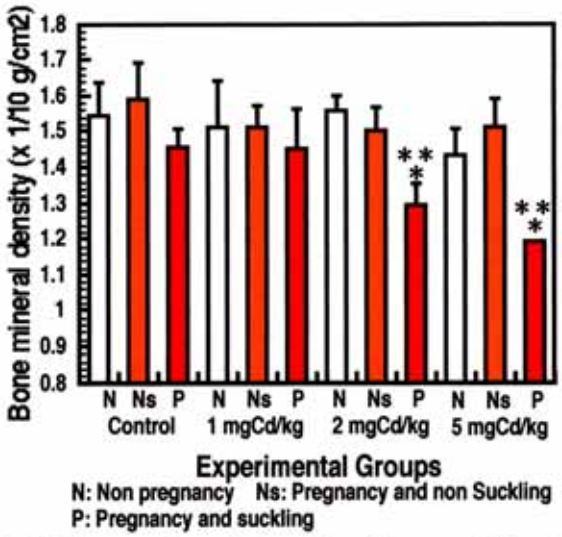


Fig. 4 Bone mineral density of femur of female rats after oral cadmium administration and pregnancy.

*: Significant to the non pregnancy of each group at p<0.05 by ANOVA-PLSD test.

**: Significant to the pregnancy of control group at p<0.05 by ANOVA-PLSD test.

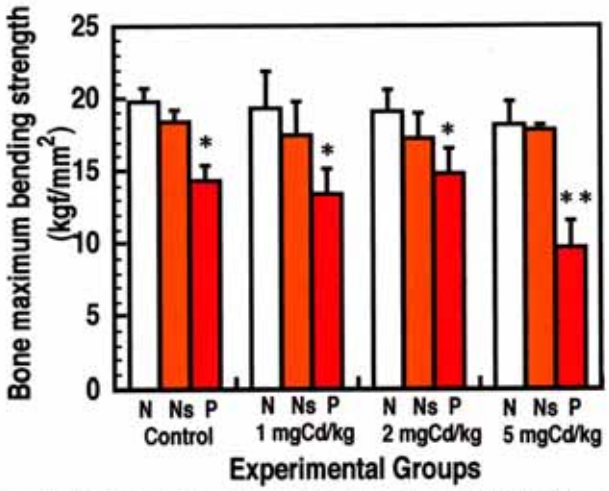


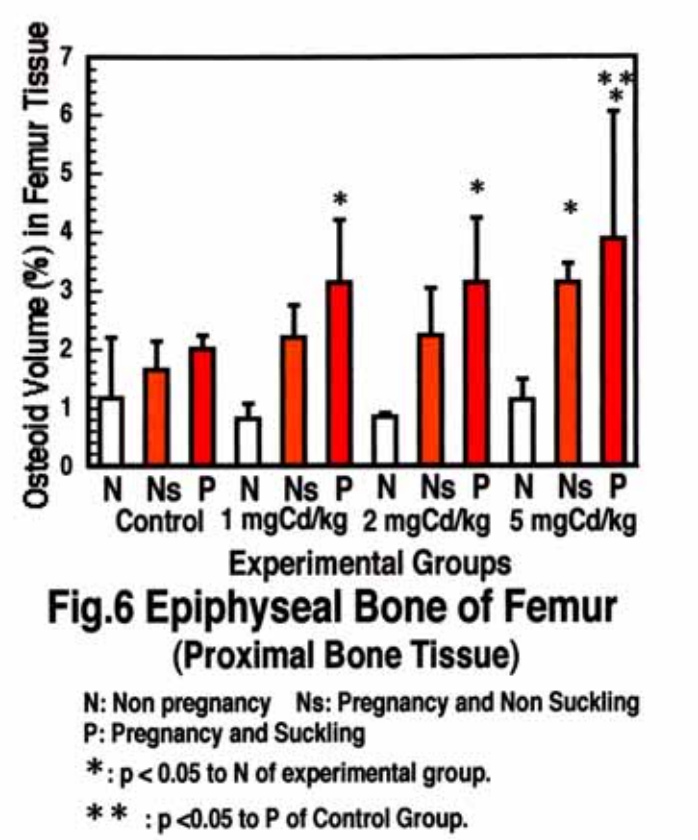
Fig.5 Bone maximum bending strength of femur after oral cadmium administration and gestation-lactation in female rats.

N: Non pregnancy Ns: Pregnancy and Non Suckling
P: Pregnancy and Suckling

*: $p < 0.05$ to N of control group.

**: $p < 0.05$ to P of control group.

The decreased bone maximum bending strength caused by Cd intake together with lactation load was more prominent and sensitive than the case of bone mineral density (Fig. 4&5). And the positive correlation was found between these indexes of bone.



Osteoid of femur tissue stained by Yoshiki method was measured osteoid rate (Fig.6).

The osteoid volume (%) in femur tissue increased with lactation load. Moreover, the osteoid volume of the groups of Cd intake with lactation load increased significantly depending on increasing of intake and accumulation of Cd in femur. Particularly, in the 5mg Cd/kg group, the osteoid volume increased significantly compared with the osteoid volume by lactation load of control group. Urinary excretion of DPyr reflecting bone absorption increased significantly by lactation load of all groups including the control group. The prominent increase depending to Cd intake of urinary DPyr excretion was not found (Fig.7). It was thought that bone resorption was activated by lactation after gestation. Because the urinary excretion of DPyr increased significantly in the all groups of lactation load including the control group. However, the dose dependent increase to Cd intake was not recognized.(Fig. 7) The alteration of bone resorption index was more prominent with DPyr than Pyr. On the other hand, the significant alteration of plasma osteocalcin level, which is biochemical index of bone formation, was not found with all Cd intake group (unpublished data).

Activity of plasma vitamin D (1 alpha -25(OH) 2-D) increased with gestation and delivery. However, the activity was decreased by lactation load and Cd intake. Particularly, the vitamin D activity showed meaningful degradation in the control group of non-pregnancy of Cd intake of 5mgCd/kg. (unpublished data) Such as reflecting the decrease of bone mineral density and of bone maximum bending strength, the urinary excretion of calcium increased (Fig.8).

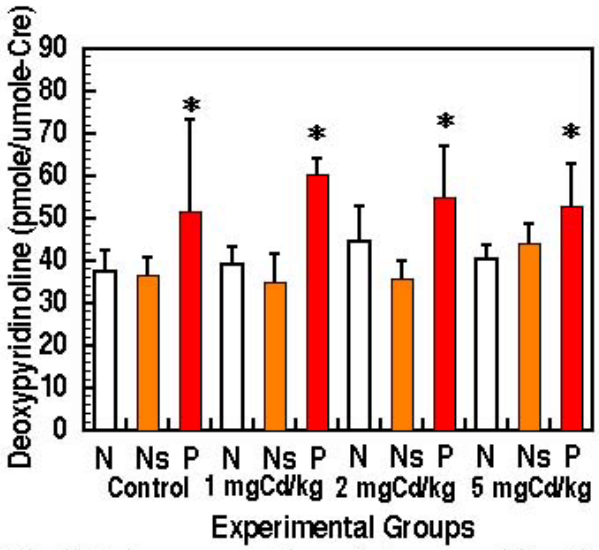


Fig. 7 Urinary excretion of deoxypyridinoline.

N: Non pregnancy Ns: Pregnancy and Non Suckling
P: Pregnancy and Suckling
*: $p < 0.05$ to N of Control.
**: $p < 0.05$ to P of Control.

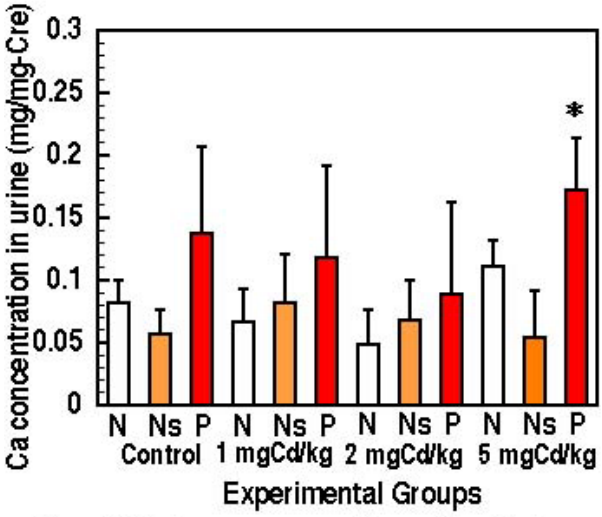


Fig. 8 Urinary excretion of calcium.

N: Non Pregnancy Ns: Pregnancy and Non Suckling
P: Pregnancy and Suckling
*: $p<0.05$ to N of Experimental group.
**: $p<0.05$ to P of Control group.

As for the bone metabolism disorders, the Cd intake and lactation load are important factors on the enhancement of bone resorption and the ossiferous depression. In the case of 1 mgCd/kg group by which the intestinal tract absorptivity of Cd is an average of 0.5% with 0.4-0.7 % is presumed to be daily Cd intake of Japanese (Yoshida et al., 1998; Ohta et al., 1998, 2000, 2002a). Especially the prominent Cd effect to the renal function and bone

metabolism was not observed in the 1mg Cd/kg group. The increasing tendency of urinary excretion of Ca and P was also observed in the groups of 2 and 5 mg Cd/kg. In such a situation, the significant decrease of bone mineral density and of bone bending strength caused by Cd intake together with lactation was found especially in mother rats of the 5 mg Cd/kg group. The osteoid volume of femur and urinary Ca excretion increased significantly in the 5 mg Cd/kg group. (Fig. 6&8) Namely, it was suggested that the bone absorption was promoted and the bone formation was depressed in bone metabolism disorder caused by Cd intake together with lactation load.

From the results mentioned above, it was thought that the disorder in bone metabolism caused by lactation load and Cd exposure are based on accentuation of bone absorption, and suppression of bone formation.

Discussion

In experimental research and epidemiological survey about the evaluation of Cd toxicity, so far, there is much results carried out by the surgical management and by comparatively high Cd dose injection (Hiratsuka et al., 1997; Sacco-Gibson et al., 1992; Katsuta et al., 1993). However, there is only a little study investigating about the health effects by daily Cd intake level of human in a more normal physiological condition. Particularly, the detailed study to evaluate the effects of low-level daily Cd intake under normal physiological condition considering the pregnant-lactation has been not reported sufficiently. There is only a few research reports in which the health effect of Cd was evaluated considering the chemical form of Cd in intestinal tissue and the tissue distribution of Cd in the case of low-level Cd intake condition (Groten et al., 1991,1994; Ohta et al., 2000; Noel et al., 2004). So far, there are many accumulated research data of model simulation study such as the parenteral administration or surgical management which was already aimed for problem solving by comparatively a large quantity of Cd exposition. From the evaluation of health effect of Cd by the conventional acute poisoning, it is thought that it is difficult to evaluate health effects of low-level daily Cd intake exposure. As the dose of Cd intake becomes lower, the presence of intestinal tissue of metallothionein becomes more important for the distribution and the toxic effect of Cd (Ohta and Cherian, 1991; Ohta et al., 2000). The systematic study for the health effect of low-level daily Cd intake based on the dose-effect and the dose-response relationship is insufficiency. For the setting of criteria, the suggested criteria value by FAD/WHO combination food standard committee (CODEX) Joint FAO/WHO Codex Alimentarius Commission is controversy at the present.(WHO, 1989a, 1989b,1993; Satarug and Moore, 2004). After all, the index of health effect evaluation and the significance and the setting of criteria may be indistinct at the present time and an agreement is not obtained. The setting of criteria based on health risk evaluation by Cd intake and the epidemiological study of health effects by daily Cd intake is expected. For an evaluation index criteria of renal function aberration, for example, such as B2-MG, we do not get the unified in setting of the evaluation

(Kido et al., 1993; Nishijo et al., 2004; Honda et al., 2003; Ikeda et al., 2000; Jarup, , 1994, 2000, 2002; WHO IPCS, 1992; Satarug et al., 2003, 2004). The present guideline for the decision of Cd nephropathy with quantity of urinary excretion of β2-MG is still argued. So far, there are many studies for renal damage and bone disorder caused by relatively high Cd dose considering the case of itai-itai disease. However, now the detailed study is needed for the health effect relating with the daily Cd intake of human. Accordingly, the experimental study is required more to be able to extrapolate the case of human from animal experiment based on Cd intake considering the daily Cd intake level of human. In that case, many factors such as a food nutritional factor and such as the alterations of distribution and health effects caused by modification of the chemical configuration of Cd by MT in intestinal absorption stage of Cd must be considered naturally.

Recently, an energetic study has been reported about bone metabolism disorder onset by low-level Cd exposure (Bucher et al., 1990;Jarup et al.,1998, Alfven et al., 2000; 2002; Brzoska and Moniuszko-Jakoniuk, 2004; Brzoska et al., 2004; Satarug and Moore, 2004; Jin et al.,2004; Stassen et al., 1999; Yamanaka et al., 1998). Bone metabolism is very sensitive, and various factors seem to affect. In particular in female animal, pregnant childbirth and nursing (breast milk / lactation) are very serious physiological load to mother's body. Also, it has been reported that the decrease by nursing of bone mineral density of femur in mother rats significantly decreased dose-dependently by chronic Cd intake of low concentration. Namely in this study, it was found that low -level Cd intake which is estimated around 2-5 times of daily Cd intake level of human caused the decreased bone mineral density and the renal dysfunction in addition of the load of gestation and nursing (Fig. 2 and 4,5,6). The sensitive bone metabolism of mother rats is also necessary to be considered including interaction of various nutritional factors and Cd. The health effects to mother by pregnancy and nursing are massive, and renal dysfunction (pregnancy toxemias) and bone metabolism disorders (osteoporosis after pregnant delivery) are well known. It is also known that this osteoporosis recovers by the nutritive supplying such as calcium appropriate afterwards (Ohta et al., 2002a). As for this, many maternal calcium is caused by being shifted to a newborn infant by lactation. Cd compete with calcium in calcium absorption from an intestinal tract (Fig. 9 from Ohta et al., 2002a; Epstein, 1988; Delmas, 1993).

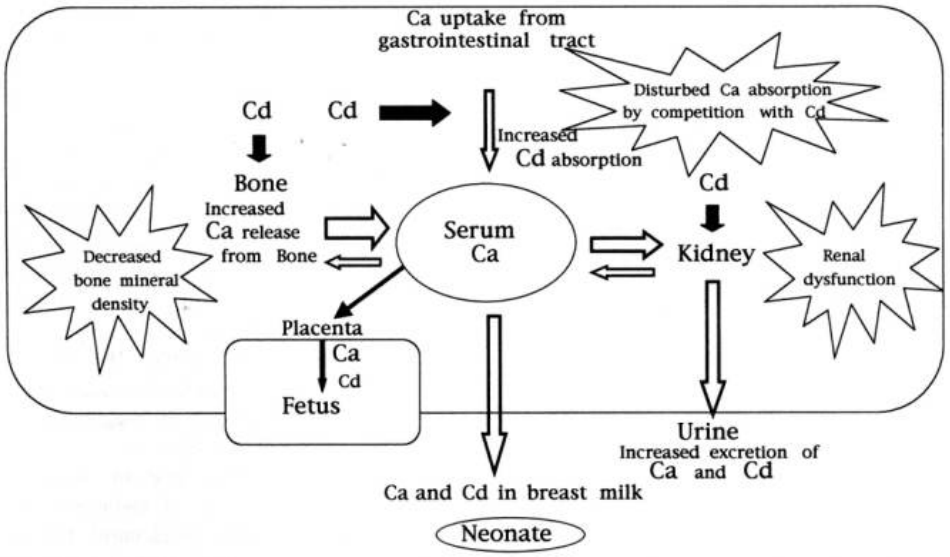


Fig.9. Schematic illustration of Cd effects on Ca metabolism during lactation.

In addition, it is well known that an intestinal absorption of Cd is increased by the depletion of calcium and iron. From these things, it is thought that the exposure of Cd affects restrainingly on the absorption of calcium from an intestinal tract in gestation and nursing. Namely, it is understand that the renal dysfunction and the bone metabolism disorder caused by Cd exposure and by calcium metabolism disorder.

In this study, the decrease of bone density was caused by lactation, but it was clarified that the decreased bone density became more remarkable by additional Cd intake in the group of 5 mg Cd/kg. However from the results in this study, the bone mineral density of the group (1 mg Cd/kg/day) corresponded with daily Cd intake level of human was not the significant difference compared with that of control group. From these findings, it was suggested that the setting of the acceptable daily intake level of Cd requires the consideration with importance of nursing load as well as with smoking habit and nutritional status relating with bone metabolism, because the bone mineral density decreased significantly by Cd intake of around 2 times (7-8 ug/kg/day degree) of daily Cd intake of human. The urinary excretion of amino acid and NAG and β 2MG increased around 20- 40ug/g significantly. At this time, renal Cd concentration was not changed relating with pregnant and nursing load. This is remarkably lower concentration in comparison with 200ug/g of conventional critical renal Cd concentration (WHO IPCS, 1992; ILZRO, 1989; Nordberg, 1999). It is thought that the issue for evaluation of the effect of Cd intake from such results to bone metabolism by pollution of conventional Cd is shifting into the problem of health effect by Cd intake level in our daily life.

In the general population in Sweden and in Belgium whose environmental Cd exposure

levels, a decrease in bone mass related to urinary Cd has been reported recently.(Satarug and Moore, 2004). New data have emerged suggested that also relatively low cadmium exposure may give rise to skeletal damage, evidenced by low bone mineral density (osteoporosis) and fractures. Several reports have shown that renal damage and/or bone metabolism disorder are likely to occur at lower renal cadmium levels (Jarup, 2002; Satarug and Moore, 2004;Brzoska and Moniuszko-Jakoniuk, 2004; Nishijo et al.,, 2004). European studies have shown signs of cadmium induced kidney damage in the general population at urinary cadmium levels around 2-3 μg Cd/kg creatinine.

We compared the effects of gestation, lactation load and Cd intake to renal function and bone metabolism in mother rats in this study. In the mother rats of 5 mg Cd/kg group, Cd affection to bone metabolism was resulted prominently after the lactation load for 4 weeks. But without the lactation load, this decreased bone mineral density by Cd intake was not significant compared with that of control mother rats. It was clarified that the lactation load affected physiologically the bone metabolism of mother rats. Moreover, it was also suggested that Cd intake (5 mg Cd/kg) had additional effect with lactation load to the bone metabolism of mother rats. The Cd intake of 5 mg Cd/kg group was estimated as about 5-fold amount of the daily Cd intake of Japanese. However, the concentration of Cd in kidney of 5 mg Cd/kg group was much more less than the value of 200 μg/g suggested for the critical concentration. On the other hand, in the 1 mg Cd/kg group, a similar level with the daily Cd intake of Japanese, these prominent effects to renal function and to bone metabolism by Cd were not found. It was thought that the critical concentration of Cd effects may be changed according to the condition of Cd exposure and the physiological condition of female rats such as pregnancy and lactation load as well as the case of the condition of Cd intake such as the duration and dose of Cd intake. From these results, it was thought that these factors such as lactation and the Cd intake level must be considered for the reevaluation of an acceptable daily Cd intake amount and of the critical concentration of Cd toxicity in bone metabolism, the target organ. Particularly, it was suggested that the detail study of Cd effect to mother in human under normal physiological body load such as pregnancy, delivery, and lactation need to reevaluate the daily Cd intake amount and Cd toxicity in human. Namely, the low-level Cd intake together with lactation load affect bone metabolism and be an important target organ of adverse health effect by Cd. From these results, it was concluded that both a low level Cd intake together with lactation was more affective to the renal function and bone metabolism of mother rats (Staessen et al., 1999; Ohta et al., 2000, 2002b; Alfven et al., 2002).

The mechanisms behind Cd-induced bone damage are not clear, but the possible mechanism is implicated Cd interaction relating with calcium metabolism,and with calciuria and/or dysfunction of tubular cells leading to decreased Ca absorption from the gastrointestinal tract. Both experimental and epidemiological data indicate that Cd can affect on bone metabolism before the manifestation of renal damage(Ohta et al., 2000; Ogoshi et al., 1989). Cd also interferes with trace metals acting in bone mineralization, mainly zinc, iron,

and copper. Previously described interactions involving various mineral deficient diets have been reported in animals; for instance in both zinc and iron deficiencies, Cd uptake by tissue increased in those reports. The effects of essential minerals on Cd accumulation and toxicity have been also investigated in vivo by feeding Cd diets supplemented with various minerals; the most protective effect against Cd accumulation was observed with a supplement combining calcium, phosphorus, iron and zinc (Ohta and Cherian, 1995; Ohta et al., 2002). All these essential minerals interacting with Cd play a role in bone turnover. As the result, the chronic exposure to environmental Cd may disturb essential mineral metabolism at low doses. Besides interfering with calcium metabolism, the Cd effect on trace elements metabolism might help to explain the risk of Cd associating on osteoporosis and osteomalacia.

In conclusion, it was suggested that the maternal condition such as lactation load should be considered, to evaluate the effect of low -level Cd intake on renal function and bone metabolism, together with the Cd intake conditions (such as dose, chemical form of Cd, nutritional factors, etc.). The detail study considering the case of daily Cd intake of human to describe the relationship of dose-effect / dose-response of Cd is needed to evaluate the effects of low level Cd intake.

Acknowledgments

This study was supported by several grants from the Ministry of Education, Culture, Sports, Science and Technology (C(2), # 16590481), and from the Japan Public Health Association (Health Sciences Research Grant on Environmental Health), and from the grant of Kitasato University School of Allied Health Sciences (Grant-in-Aid for Research Project, 2004). We would also like to thank Mr.Hiroki Fujita of Nippon Dental College for his technical assistance with pathology.

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[WS-9]

MODIFICATION OF CADMIUM TOXICITY BY COBALT AND MANGANESE

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In a previous study, we have established cadmium-resistant metallothionein-null (Cd-r) cells from embryonic fibroblast cells derived from metallothionein (MT) I and II knockout mice [1, 2, 3]. The primary cause of cadmium resistance in Cd-r cells was the reduced accumulation of cadmium in cells, which was conferred by the low rate of cadmium uptake. Multi-tracer analysis demonstrated that the uptake of manganese was also reduced in Cd-r cells. Competitive inhibition of cadmium uptake by manganese was observed in parental MT-null cells, as well as in HeLa, Caco2, and PC12 cells, but not in Cd-r cells. These results suggest that manganese is involved, at least in part, in cellular cadmium uptake and this pathway is not functioning in Cd-r cells. The addition of manganese in the media decreased cadmium accumulation and toxicity in parental cells, but not in Cd-r cells. Therefore, the transport system for manganese may play a role in cellular uptake and toxicity of cadmium. Recently, the ferrous iron transporter, DMT1 (Nramp2), has been shown to be involved in cellular cadmium uptake especially in the intestinal absorption [4, 5]. However, the properties of the transport system for cadmium/manganese in MT-null cells were not similar to those of DMT1 as judged by optimal pH and competition profiles of metals. Therefore, we are now investigating the responsible gene for the cellular incorporation of manganese and cadmium into MT-null cells.

As manganese is shown to be involved in the protection against cadmium toxicity in cells, we next examined the effects of manganese on *in vivo* toxicity of cadmium in mice. Simultaneous administration of $MnCl_2$ with $CdCl_2$ showed significant reduction of acute cadmium toxicity as determined by plasma GPT activity and testicular hemorrhage. However, the accumulation of cadmium in the liver, kidney, and testis was not influenced by co-administration of manganese. To explore the mechanism of manganese protection against cadmium toxicity, we examined the effects of co-administration of other metals with cadmium. Among the metals examined, only manganese and cobalt showed significant reduction of plasma GPT and testicular hemorrhage caused by cadmium administration. The tissue accumulation of cadmium was not affected by cobalt as well as by manganese, suggesting that the other mechanism than cadmium incorporation into cells is involved in the protection against *in vivo* cadmium toxicity by manganese and cobalt. As manganese is a weak MT inducer and cobalt is not considered as an MT inducer, we examined the effects of these

metals in MT null mice. The protective effects of manganese on acute cadmium toxicity were partly reduced in MT-null mice but those of cobalt were not, suggesting that the protection by cobalt may be independent of MT synthesis.

Since inflammatory cytokines are known to be involved in liver injury and several reports have suggested that some metals can modify the production of cytokines, we next examined whether the production or inhibition of cytokines are involved in the modification of acute cadmium toxicity by manganese and cobalt in mice. We also determined plasma levels of acute phase protein, serum amyloid A (SAA), which is induced by cytokines in the liver when inflammation occurred. Among inflammatory cytokines examined, only IL-6 increased in the plasma after the administration of manganese and cobalt. The peaks of plasma IL-6 levels were observed at 6 h and 3 h by manganese and cobalt, respectively. Since MT production is also involved in the protection against cadmium toxicity by manganese, we focused on the effects of cobalt on cytokine production. Cadmium administration itself also increased plasma levels of IL-6 and SAA. Co-administration of cobalt dose-dependently reduced the cadmium-induced increases in plasma GPT activity and SAA levels (Fig. 1). The reduced SAA levels in plasma reflected the reduced mRNA levels of SAA1 in the liver as measured by quantitative RT-PCR (Fig. 2). However, co-administration of cobalt with cadmium enhanced IL-6 production both at protein levels and mRNA levels (Fig. 3). On the other hand, cadmium-induced production of TNF-α was reduced by co-administration of cobalt (Fig. 4). Therefore, it is suggested that cobalt reduced the production of TNF-α caused by cadmium, and consequently protected against hepatotoxicity and SAA production.

In conclusion, manganese can inhibit cadmium uptake in some cell lines possibly via the mechanism independent of DMT1. Co-administration of manganese protected against acute cadmium toxicity in mice, but the evidence showing cadmium transport is involved was not provided. In addition to manganese, co-administration of cobalt protected against acute cadmium toxicity, which is independent of MT production. The modification of cytokine production was implicated as a new mechanism underlying the protection against cadmium toxicity by cobalt.

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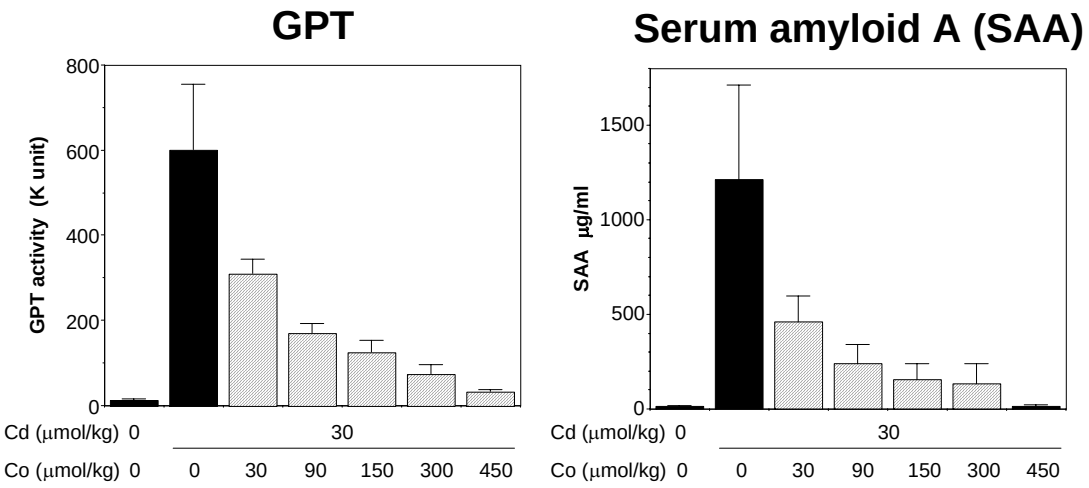

Figure 1
Co-administration of $CoCl_2$ dose-dependently reduced Cd-induced increases in GPT activity and serum amyloid A (SAA) in mice.

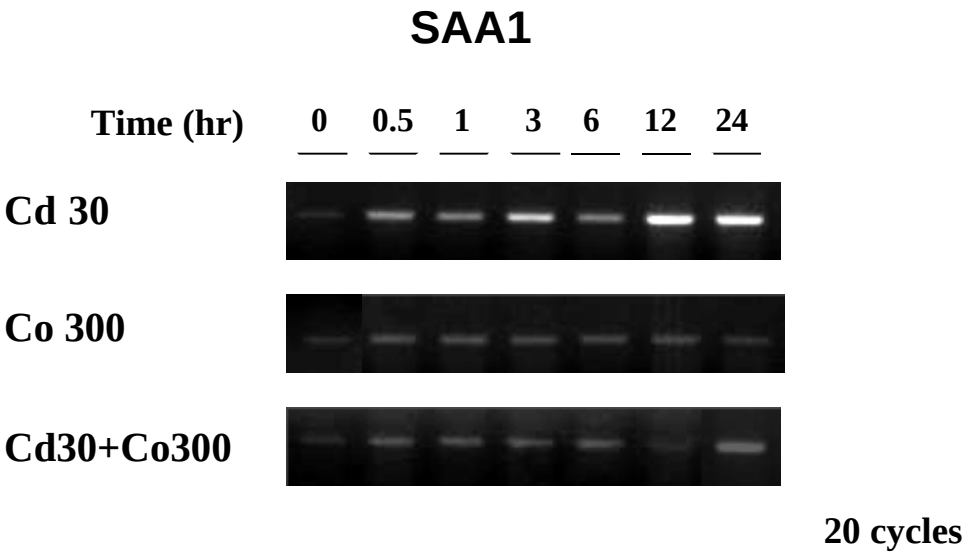

Figure 2
Quantitative RT-PCR showed that Cd-induced increases in SAA1 mRNAs in the liver of mice were markedly suppressed by co-administration of $CoCl_2$. Treatment with $CoCl_2$ alone did not induce SAA1 expression. These data reflected the protein levels of serum amyloid A.

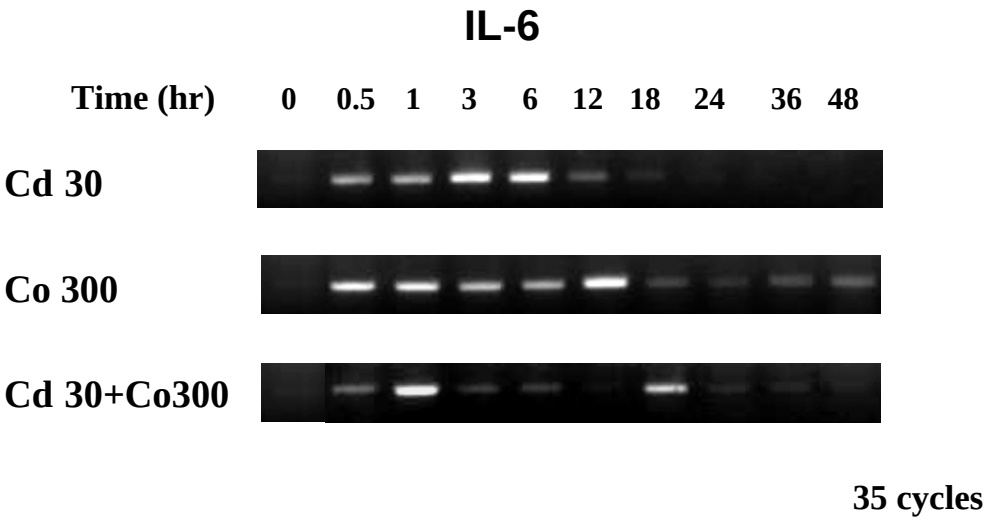

Figure 3
Quantitative RT-PCR showed that treatment with $CdCl_2$ (30 μmol/kg) alone, $CoCl_2$ (300 μmol/kg) alone, or both metals caused time-dependent increases in IL-6 mRNAs in the liver of mice, suggesting that the changes in SAA levels in mice treated with $CdCl_2$, $CoCl_2$ or both could not be explained by the changes in IL-6 expression.

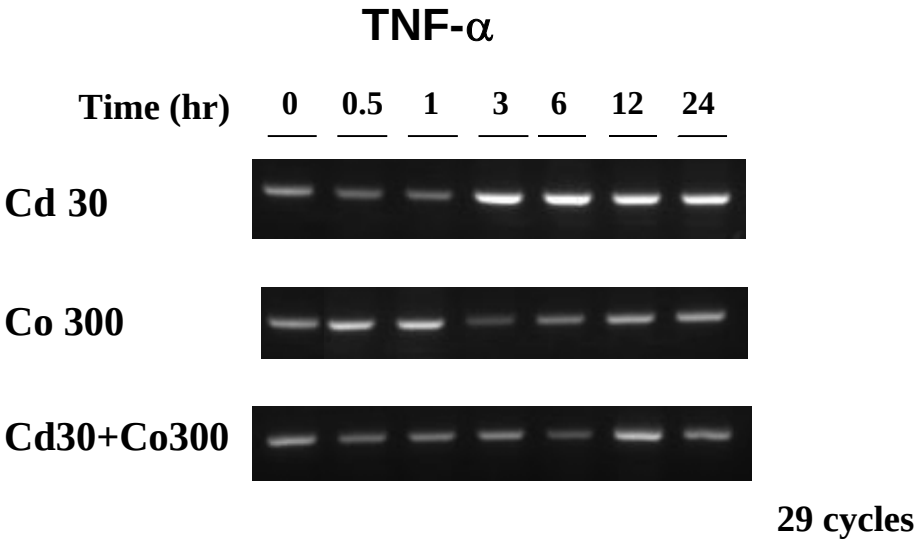

Figure 4
Quantitative RT-PCR showed that Cd-induced increases in TNF-α mRNAs in the liver of mice were markedly suppressed by co-administration of $CoCl_2$, suggesting the role of TNF-α in SAA1 expression.

[WS-10]

ROLE OF METALLOTHIONEIN IN DISTRIBUTION OF CADMIUM

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Cadmium (Cd) is a wide-spread environmental pollutant that causes various toxic effects such as kidney damage and lung carcinogenesis in humans and experimental animals. Cd accumulates rapidly in the liver and kidney of experimental animals after the Cd injection. On the other hand, metallothionein (MT) is a cysteine-rich low molecular weight protein with a high affinity for metals such as Cd, mercury, zinc and copper, and it is induced by these metals and many other factors such as glucocorticoids and cytokines. It is well known that MT is a protective factor against the Cd toxicity. Moreover, MT plays a role in the retention of Cd in the liver of mice but not in the uptake of this metal after single injection of Cd. However, the role of MT in the distribution of Cd in the gestational or long-term exposure to Cd is unclear. To clarify this issue, Cd accumulation in the tissues after gestational or long-term exposure to Cd using MT-I and II knock-out mice (MT-I/II null mice) and background-matched wild-type mice (wild-type mice) were determined.

MT-I/II null pregnant mice and wild-type pregnant mice were given 50 ppm Cd as drinking water during gestation. Cd concentration in the maternal kidney of MT-I/II null pregnant mice significantly decreased compared with that of wild-type pregnant mice, whereas Cd concentrations in the maternal liver, small intestine and brain were not different between MT-I/II null pregnant mice and wild-type pregnant mice. In contrast, Cd concentrations in the placenta, fetal liver and fetal brain of MT-I/II null pregnant mice were significantly higher than those of wild-type pregnant mice.

Next, female MT-I/II null mice and wild-type mice were given 10 ppm Cd as drinking water for 360 days. The long-term exposure to Cd markedly decreased Cd concentrations in the kidney and liver of MT-I/II null mice in comparison to those of wild-type mice. However, Cd concentration in the brain of MT-I/II null mice was significantly higher than that of wild-type mice.

In the present study, we found that MT affects on the distribution of Cd in various tissues in gestational or long-term exposure to Cd. In the gestational exposure to Cd, MT plays an important role in retention of Cd in the maternal kidney, and prevention of Cd accumulation in the placenta and fetus. In the long-term exposure to Cd, MT also plays a major role in retention of Cd in the kidney and liver, and prevention of Cd accumulation in the brain.

[WS-11]

THE ROLE OF AMINO ACID TRANSPORTERS IN THE METHYLMERCURY TRANSPORT

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Methylmercury is widely known for its potent neurotoxicity and the causal substance of Minamata disease. Since the conjugates of methylmercury with thiol compounds are easily formed *in vivo*, the metabolism and transport of glutathione, cysteine and their derivatives are important determinants of the absorption, tissue distribution and elimination of methylmercury. It has been proposed that the amino acid transport system L, which transports large neutral amino acids and let the methylmercury-cysteine conjugate permeate plasma membrane of cells, is one of the major routes for methylmercury mobilization in the animal body (Fig. 1) (1, 2). Because of the lack of knowledge on the molecular nature of system L amino acid transporters, it bas been difficult to investigate the molecular mechanisms of absorption and distribution of methylmercury in the body and the role of transporters in the manifestation of methylmercury cytotoxicity.

We previously identified two isoforms of system L transporters LAT1 (L-type amino acid transporter 1) and LAT2 (L-type amino acid transporter 2) (3, 4). For the plasma membrane expression, LAT1 and LAT2 require additional protein 4F2hc (4F2 heavy chain; CD98) with a single membrane-spanning domain. LAT1 and LAT2 form functional heterodimeric complex with 4F2hc via a disulfide bond. LAT1 is primarily expressed in tumor cells and transports preferentially large neutral amino acids. LAT2 is an epithelial type transporter with the substrate selectivity to cover all the neutral amino acids (5). By generating specific antibodies against LAT1, LAT2 and their accessory subunit 4F2hc, we demonstrated that these proteins are expressed in the brain capillary endothelial cells and syncytiotrophoblast of the placenta, indicating that both LAT1 and LAT2 are present at blood-brain barrier and placenta barrier and function as a permeation path for neutral amino acids (Fig. 2) (2, 5, 6).

In order to test the hypothesis on the system L-mediated methylmercury transport, we examined whether methylmercury and its cysteine-conjugate are transported by LAT1 and LAT2 expressed in *Xenopus* oocytes. Human LAT1 or human LAT2 were expressed in *Xenopus* oocytes together with their accessory subunit 4F2hc by injecting their cRNAs. We synthesized [^{35}S]methylmercury-cysteine conjugate and examined whether methylmercury and its cysteine-conjugate are transported by LAT1 and LAT2. Both LAT1 and LAT2 transported [^{35}S]methylmercury-cysteine conjugate in a concentration-dependent manner, whereas [^{14}C]methylmercury was not accepted by the transporters (Fig. 3) (2). Because

methylmercury-cysteine conjugate and leucine competed the same binding sites of LAT1 and LAT2, we concluded that methylmercury is transported as a cysteine-conjugate through system L transporters LAT1 and LAT2.

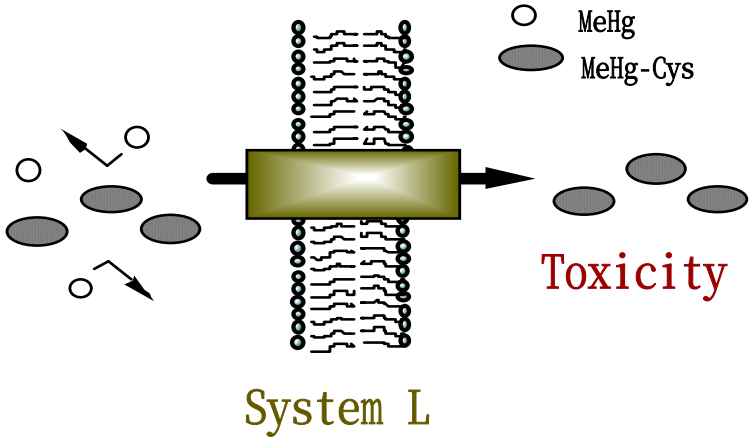

Fig. 1: Role of system L in the permeation of methylmercury through the plasma membrane. Methylmercury can pemetrate the plasma membrane as a cysteine conjugate through system L and exerts its cytotoxicity.

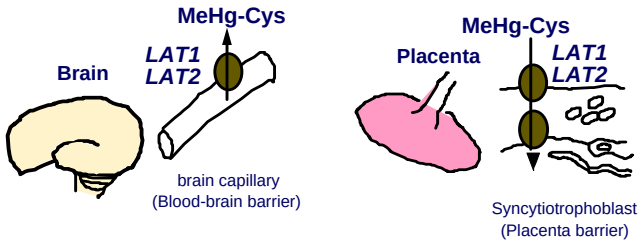

Fig. 2: Permeation of methylmercury through the blood-tissue barriers. System L transporters LAT1 and LAT2 are present at the blood-tissue-barriers such as blood-brain barrier and placenta barrier and mediate the transport of methylmercury- cysteine conjugate .

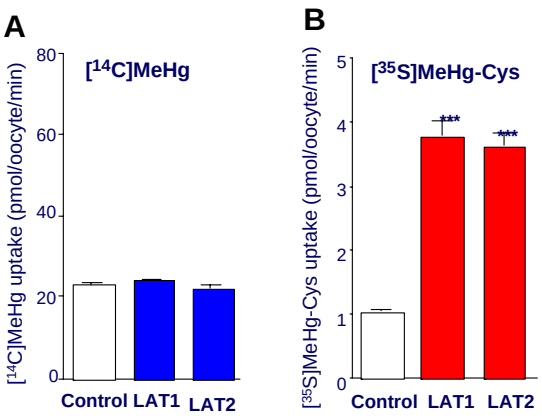

Fig. 3: Transport of methylmercury-cysteine conjugate by system L transporters LAT1 and LAT2. The transport of [^{14}C]methylmercury (A) and [^{35}S]methylmercury-cysteine conjugate (B) were measured on LAT1 and LAT2 expressed in *Xenopus* oocytes with their accessory subunit 4F2hc. Both LAT1 and LAT2 transported [^{35}S]methylmercury-cysteine conjugate, whereas [^{14}C]methylmercury was not accepted by the transporters

In order to demonstrate the significance of system L transporters in the manifestation of methylmercury-cytotoxicity, we have examined the effects of a classical system L inhibitor BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid) on the toxicity of methylmercury in T24 human bladder carcinoma cells which express LAT1 at high level. In T24 cells, the uptake of methylmercury-cysteine conjugate was almost completely inhibited by BCH, indicating that the transport of methylmercury-cysteine conjugate in T24 cells is largely mediated by system L (7). We found that the viability of T24 cells was much lower in the presence of methylmercury-cysteine conjugate than that in the same concentration of methylmercury-Cl. Furthermore, BCH significantly reduced the toxicity of methylmercury-cysteine conjugate on T24 cells, whereas it had no effect on the cytotoxicity of methylmercury-Cl (Fig. 4). Therefore, we concluded that the cytotoxicity of methylmercury is mediated by system L transporters. In addition, we have generated new high-affinity inhibitors for system L transporters with the affinity ~1,000 times higher than that of BCH. In order to evaluate these inhibitors, we have examined their effect on the methylmercury toxicity elicited by methylmercury-cysteine conjugate in T24 cells. We have found that the high-affinity inhibitors KYT0193 and KYT0206 significantly reduced the toxicity of methylmercury-cysteine conjugate, suggesting that the high-affinity inhibitors could be used more efficiently to prevent methylmercury-toxicity.

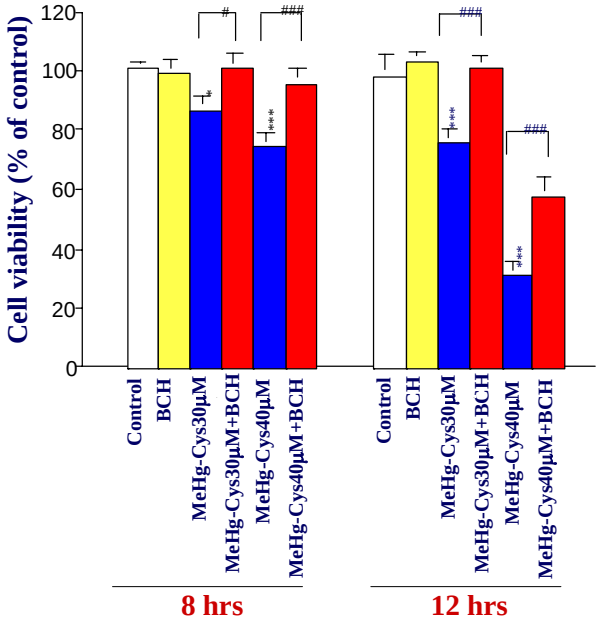

Fig. 4: Protection against cytotoxicity of methylmercury-cysteine conjugate by a system L inhibitor. The cytotoxicity induced by 8 hr or 12 hr treatment of T24 human bladder carcinoma cells with methylmercury-cysteine conjugate (30 microM or 40 microM) was suppressed by system L inhibitor BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid).

We have, furthermore, cloned additional system L transporters LAT3 (L-type amino acid transporter 3) and LAT4 (L-type amino acid transporter 4) which are structurally distinct from LAT1 and LAT2 (8). In contrast to LAT1 and LAT2, LAT3 and LAT4 did not transport methylmercury as its cysteine-conjugate form. It is, thus, concluded that system L transporters LAT1 and LAT2 are the major transporters responsible for the mobilization of methylmercury in the animal body and for the manifestation of the toxicity of methylmercury-cysteine conjugate. Recently, we have identified a novel neutral amino acid transporter *h*B^0AT1 that is expressed in the apical membrane of epithelial cells and is responsible for the absorption of neutral amino acids from the small intestine (9). We have found that methylmercury is transported by *h*B^0AT1 as a cysteine-conjugate form, suggesting *h*B^0AT1 is a transport path of methylmercury-cysteine conjugate at the apical membrane of small intestine epithelial cells (Fig. 5). The inhibition of such transporters responsible for the plasma membrane permeation of methylmercury-cysteine conjugate as LAT1, LAT2 and *h*B^0AT1 could be a new rationale to prevent methylmercury toxicity. More extensive studies should be necessary to examine whether newly developed high affinity inhibitors could be used to treat or prevent methylmercury toxicity *in vivo*.

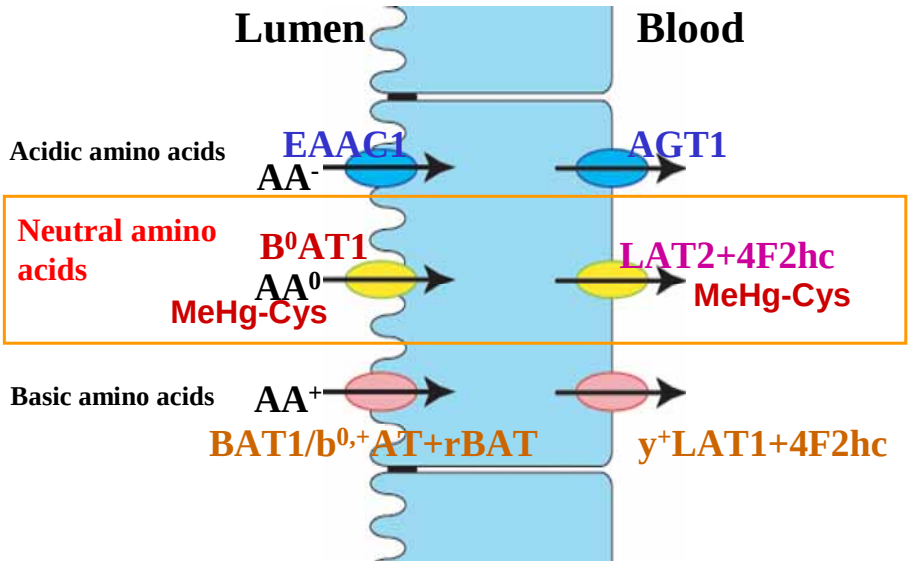

Fig. 5: Absorption of methylmercury-cysteine conjugate from the small intestine. Methylmercury-cysteine conjugate were absorbed via neutral amino acid transport systems in the small intestine. System B^0 transporter *h*B^0AT1 is responsible for the apical membrane transport, whereas system L transporter LAT2 is proposed to be responsible for the basolateral membrane transport of methylmercury-cysteine conjugate.

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[WS-12]

CELLULAR STRESS RESPONSES FOLLOWING EXPOSURE TO METHYLMERCURY

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ABSTRACT

Low levels of methylmercury (MeHg) induce apoptosis in various cell types. Apoptosis is triggered when cellular damage cannot be overcome by cell repair functions. Here the early and late events following MeHg exposure were investigated in an *in vitro* model cellular system in order to know the main cause to trigger MeHg-induced apoptosis. The model system was constructed using mouse myogenic C2C12 cell line. The susceptible cell line showed apoptosis by 24 hr after exposure to low level of MeHg. Apoptotic cells showed positive immunostaining with an anti-activated caspase 12 or an anti-activated caspase 3 antibody. Gene expression profiles and cellular stress signaling pathways were compared between these MeHg-susceptible and MeHg-resistant cell lines. The early increase in reactive oxygen species caused multigenic transcriptional changes encoding nucleo-cytoplasmic transport and protection against oxidative stress. Time course of the activation of cellular stress signaling pathways and failure in the expression of ER stress-related proteins at the early stage confirmed that oxidative stress plays a central role in MeHg-cytotoxicity. The activation of caspase 12 and caspase 3 was suppressed by the co-addition of antioxidant, Trolox. Higher oxidative stress in MeHg-susceptible cell line resulted in the sustained activation of the ASK1 and SAPK/JNK pathways. The results indicate that MeHg-induced early oxidative stress causes multigenic transcriptional changes and the failure in the protection against the early oxidative stress triggers the following apoptosis pathways.

Key words: methylmercury, apoptosis, oxidative stress, ASK1, SAPK/JNK, caspase 12, ER stress

Low levels of methylmercury (MeHg) induce programmed cell death (apoptosis) in various cell types including neural origin, glial origin, myogenic origin, microglia, monocytes and lymphpoid cells. We reported MeHg-induced apoptosis in myogenic cells, which were

constructed in C2C12 cells stably transfected with myotonin protein kinase (DMPK) cDNAs with CTG repeats (Usuki et al, 1997; Usuki and Ishiura, 1998; Usuki et al., 2000). We demonstrated that these transformants show CTG repeat number-dependent susceptibility to MeHg cytotoxicity (Usuki et al., 2000). Mutant DMPK cDNA-transformants containing 160 CTG repeats (C2C12-DMPK160) show apoptotic cell death by 24 hr after exposure to low levels of MeHg. In contrast, wild-type DMPK cDNA-transformants containing 5 CTG repeats (C2C12-DMPK5) show resistance to the same concentration of MeHg. Apoptosis is triggered when cellular damage cannot be overcome by cell repair function. Here the early and late events following MeHg exposure were investigated using this *in vitro* model cellular system in order to know the main cause to trigger MeHg-induced apoptosis.

First we investigated the involvement of caspases in MeHg-induced apoptosis in this myogenic cell lines. So far the involvement of caspases in MeHg-induced apoptosis has been reported as positive (Nishioku et al., 2000; Belletti et al., 2002) and negative (Dare et al., 2001A; Dare et al., 2001B). Immunocytochemistry with an anti-activated caspase 12 antibody showed positive staining 14-16h after exposure to 0.3 μM MeHg in C2C12-DMPK160 (Fig. 1 upper panel). The positive staining was detected in apoptotic cells, indicating ER stress-induced apoptosis plays a role in the late stage of MeHg cytotoxicity (Fig. 1, lower panel). Immunostaining with an anti-activated caspase 3 antibody was also detected in apoptotic cells 14-16 h after exposure to MeHg (Fig.2). Positive immunostaining of apoptotic cells with anti-activated caspase12 and anti-activated caspase 3 antibodies in the late stage of MeHg cytotoxicity suggests that MeHg may cause apoptosis due to both ER- and mitochondria-generated processes or due to cross-talk between ER- and mitochondrial stresses.

Quantitative *in situ* ROS analysis using DCF-DA, di (6-carboxy-2'-7'-dichlorofluorescein diacetate, di) showed that levels of intracellular ROS increase beginning 2-3hrs after exposure to MeHg. Higher intracellular ROS levels in the MeHg-susceptible cell line suggest a defect in the cellular redox system in this cell line. The MeHg-induced increase in intracellular ROS concentration was clearly suppressed by the co-addition of the antioxidant Trolox, a water soluble vitamin E derivative. Co-addition of Trolox also suppressed the activation of caspase 12 and 3, suggesting that MeHg-induced early oxidative stress results in ER stress in the late stage of MeHg cytotoxicity (Fig. 3).

A time course study of the activation of cellular stress signaling pathways and the expression of ER stress-related proteins confirmed that oxidative stress plays a central role in MeHg cytotoxicity. Higher oxidative stress in the MeHg-susceptible cell line resulted in the early activation of the ASK1 and SAPK/JNK pathways. MeHg-resistant C2C12-DMPK5 cells showed the phosphorylation of ASK1 7h after the treatment with 1.0 μM MeHg. The activation of the SAPK/JNK pathways was sustained in apoptosis-susceptible cells, but poorly activated in apoptosis-resistant cells even after exposure to 1.0 μM MeHg. In contrast, the expressions of ER stress-related proteins, XBP1, ATF6, GADD 153 and GRP78 failed to

increase at the early stage following exposure to MeHg in both cell lines. The results indicate that the early event induced by MeHg exposure is oxidative stress not ER stress. The defect in the cellular responses to early oxidative stress in the MeHg-susceptible cell line triggers the processes leading to cellular damage.

DNA microarray analyses clarified that MeHg induces oxidative stress at early stages of exposure. MeHg causes multigenic transcriptional changes in cellular protective genes. We compared the early gene expression pattern of apoptosis-sensitive and apoptosis-resistant cells. Similar targeted genes were recognized 5h after MeHg exposure in these cell lines. The expressions of genes involved in mitochondrial activity and energy production were suppressed, suggesting mitochondrial stress in the early stages of MeHg cytotoxicity. At the same time, expressions of genes involved in protection against oxidative stress and glutathione metabolism increased.

Differences in the activation of cellular stress signaling pathways between the two cell lines appeared in DNA microarray analysis patterns at the late stage. DNA microarray analyses 9h after exposure to 0.4 μM MeHg clarified that the gene program into cellular damage progressed in MeHg-susceptible cell line, whereas MeHg-resistant cell line still showed transcriptional changes in genes involved in protection against apoptosis, oxidative stress and the ubiquitin proteasome system. Our results seemed to be similar to the recent transcriptional profiling analysis data following exposure to MeHg (Wilke et al., 2003).

Conclusively, early oxidative stress induced by MeHg exposure plays a central role in MeHg cytotoxicity, resulting in ER stress at the late stage. The early increase in reactive oxygen species caused multigenic transcriptional changes encoding nucleo-cytoplasmic transport and protection against oxidative stress. The failure in the protection against early oxidative stress might make the MeHg-susceptible cell line undergo apoptosis (Fig. 4). Combined treatment with inhibitors of oxidative stress and ER stress will be needed, especially at the late stage, for the protection against MeHg-induced cellular damages.

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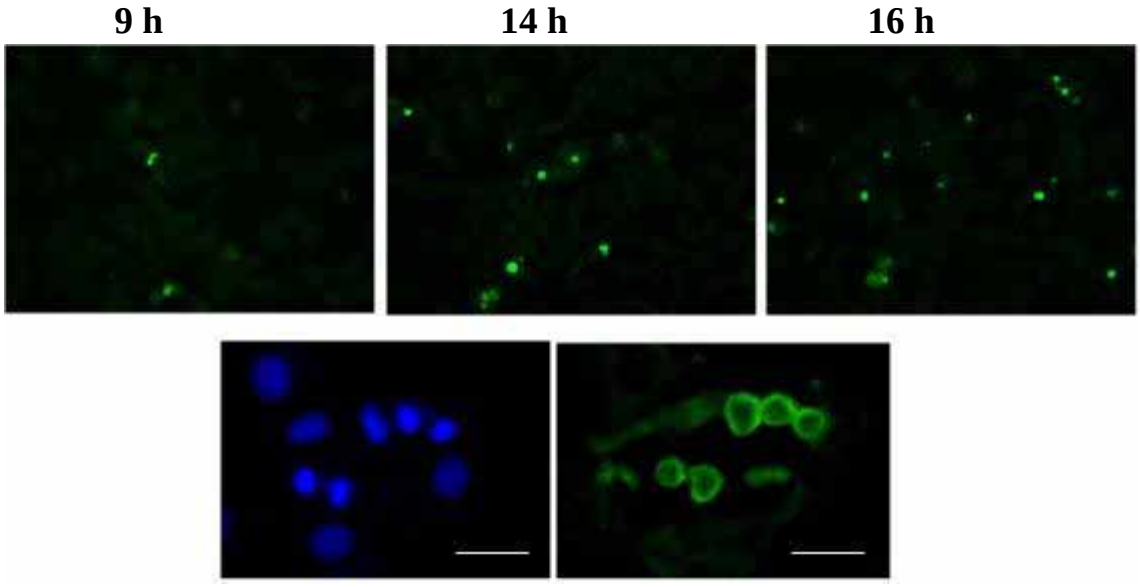

Fig. 1. Activation of caspase 12 at the late stage in MeHg-cytotoxicity

Immunocytochemistry with an anti-activated caspase 12 antibody shows positive staining14-16h after exposure to 0.3 μM MeHg in C2C12-DMPK160 (upper panel). Apoptotic cells detected by Hoechst staining (left lower panel) were positively stained (right lower panel). Bar=30 μm

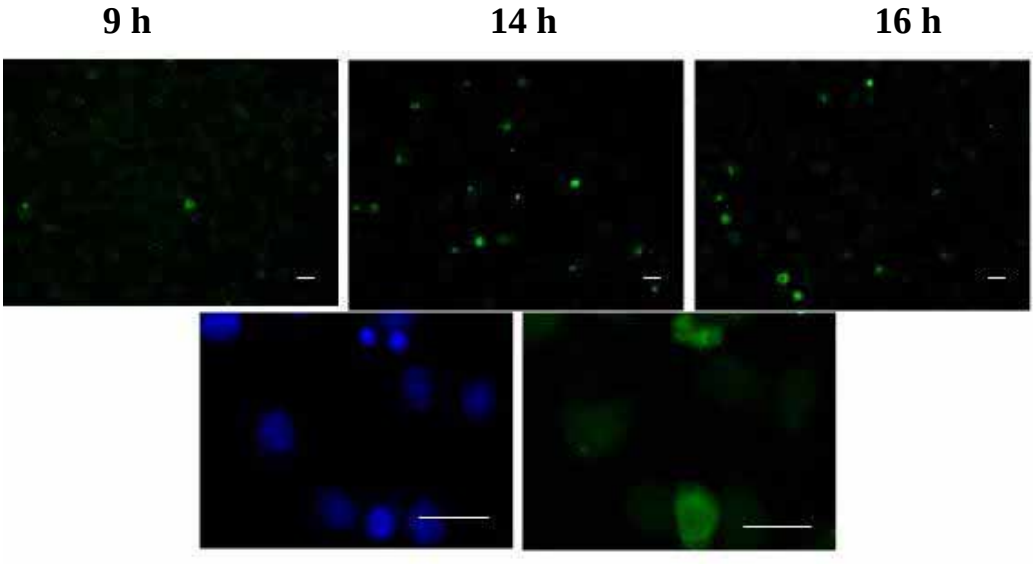

Fig. 2. Activation of caspase 3 at the late stage in MeHg-cytotoxicity

Immunocytochemistry with an anti-activated caspase 3 antibody shows] positive staining14-16h after exposure to 0.3 μM MeHg in C2C12-DMPK160 (upper panel). Apoptotic cells detected by Hoechst staining (left lower panel) were positively stained (right lower panel). Bar=30 μm

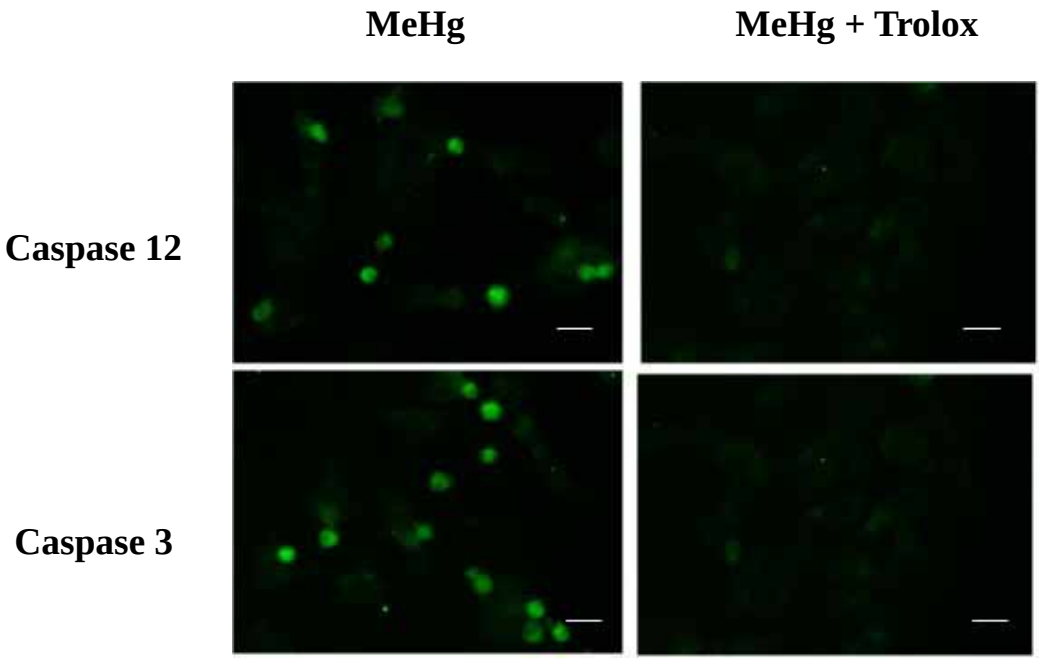

Fig. 3 Inhibitory effect of Trolox on the activation of caspases

Treatment with Trolox clearly suppressed the activation of caspase 3 and caspase 12 in C2C12-DMPK160 cells. Immunostaining data 16h after exposure to 0.3 μM MeHg are shown. Bar=20 μm

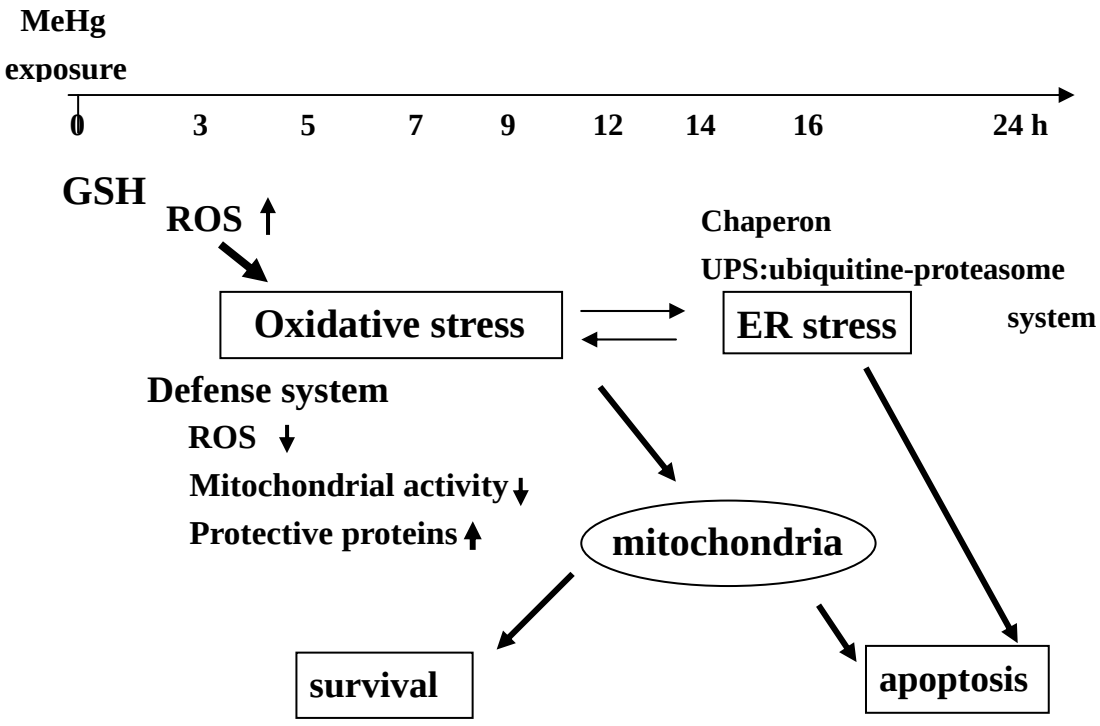

Fig. 4 MeHg-induced cellular stress responses (summary)

[WS-13]

IDENTIFICATION OF INTRACELLULAR FACTORS INFLUENCED IN SENSITIVITY OF CELLS TO METHYLMERCURY

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Abstract

We searched for novel genes that confer resistance to methylmercury in yeast (*Saccharomyces cerevisiae)* since yeast has been established as a model organism in which powerful genetic approaches can be used to elucidate fundamental but complex eukaryotic processes. We identified *GFA1* and *CDC34* as the genes involved in methylmercury resistance. *GFA1* is the gene coding for L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT), which is a catalytic enzyme involved in the production of glucosamine 6-phosphate from glutamine and fructose. *CDC34* is the gene encoding ubiquitin transferase (Ubc3), which is involved in the ubiquitination of intracellular proteins

Introduction

Considerable individual variation in the sensitivity of humans to methylmercury has been reported. Species-specific and strain-specific differences in toxic doses of methylmercury have also been observed in animals. However, the reasons for differences in sensitivity to methylmercury remain to be clarified. In cultured cells, the toxicity of methylmercury also depends on the type of cells. It seems likely that levels of expression of certain gene(s), that protect cells against methylmercury toxicity, might be involved in the differences in the sensitivity of different lines of cells to this mercury compound. Therefore, in the present study, we searched for novel genes that confer resistance to methylmercury in yeast (*Saccharomyces cerevisiae)* since yeast has been established as a model organism in which powerful genetic approaches can be used to elucidate fundamental but complex eukaryotic processes.

Results and Discussion

We investigated yeast genes obtained from a library, since yeast genes can be easily identified because the nucleotide sequences of the entire genome has been clarified. Plasmids carrying a chromosome fragment (usually containing 2-4 genes) were transfected into yeast cells, and genes contained in the chromosome fragments were expressed at high levels in the cells. Among such yeast cells, those that could grow on a medium containing

methylmercury at a concentration that would not permit the growth of normal yeast cells were selected. Since these yeast cells acquired methylmercury resistance following the introduction of the gene fragments, genes conferring methylmercury resistance must be contained in the introduced gene fragments. Plasmids were isolated from the yeast cells that had acquired methylmercury resistance, and the chromosome fragments carried in the plasmids were investigated. We identified *GFA1* [1, 2] and *CDC34* [3, 4] as the genes involved in methylmercury resistance. *GFA1* is the gene coding for L-glutamine•D-fructose-6-phosphate amidotransferase (GFAT), which is a catalytic enzyme involved in the production of glucosamine 6-phosphate from glutamine and fructose. *CDC34* is the gene encoding ubiquitin transferase (Ubc3), which is involved in the ubiquitination of intracellular proteins.

GFAT is the target molecule of methylmercury in yeast

Glucosamine 6-phosphate generated by the reaction catalyzed by GFAT is the precursor of all amino sugars synthesized intracellularly. Yeast cells cannot survive without amino sugars, because glycoproteins cannot be produced in their absence. Therefore, GFAT is an essential enzyme for the growth of yeast cells. Since GFAT is an SH enzyme, methylmercury inhibits the GFAT activity. The inhibitory effects of methylmercury on various SH enzymes were determined. The activity of GFAT was almost completely inhibited by 4 μM methylmercury, while those of other SH enzymes were hardly affected by methylmercury at this concentration. The inhibition constant (Ki) of methylmercury was 4 μM for GFAT, while the Ki values for other SH enzymes were higher than 10-fold this value. These results indicate that methylmercury has a high affinity for GFAT and specifically inhibits its activity, suggesting that GFAT is the target molecule of methylmercury.

Yeast cells transfected with the *GFA1* gene are highly resistant to methylmercury, and a relatively large amount of GFAT is synthesized by the cells. Therefore, it remains possible that strong binding of GFAT at a high concentration to methylmercury reduced the concentration of free methylmercury and suppressed methylmercury toxicity, inducing methylmercury resistance in the yeast cells. Therefore, we determined the effects of increasing the intracellular concentration of glucosamine 6-phosphate induced by the reaction catalyzed by GFAT on methylmercury toxicity. Since glucosamine 6-phosphoric acid added to media is not taken up by cells, glucosamine was added to media. Glucosamine is not synthesized by cells, but extracellularly added glucosamine is taken up by cells and transformed to glucosamine 6-phosphate by hexokinase. The toxicity of methylmercury toward the yeast cells was markedly reduced depending on the concentration of added glucosamine.

In conclusion, (1) yeast cells with high level GFAT expression are resistant to methylmercury, (2) methylmercury specifically inhibits GFAT activity, (3) methylmercury toxicity is markedly reduced by addition of glucosamine 6-phosphate, the product of the

GFAT reaction, to cells, and (4) GFAT is an essential enzyme in yeast. These results suggest that GFAT is the main target molecule of methylmercury in yeast [2].

Ubiquitin/ proteasome system as a defense mechanism against methylmercury toxicity

As described above, we showed that the gene encording Ubc3, in addition to that encording GFAT, confers methylmercury resistance on yeast. Ubc3 is an important enzyme in the ubiquitination of intracellular proteins. The ubiquitin system, which consists of a ubiquitin activation enzyme (E1), ubiquitin transferase (E2) and ubiquitin ligase (E3), is involved in the degradation of abnormal intracellular proteins. In this system, ubiquitin is activated by E1 and then binds to E2, while E3 recognizes target proteins such as abnormal proteins. E2 bound to ubiquitin binds to E3, and transfers ubiquitin to the target protein. Finally, the target protein ubiquitinated by these reactions is recognized by proteasomes and rapidly degraded.

E2 proteins belong to the family of ubiquitin transferases, and the UBC domain is preserved as the catalytic domain in all E2 proteins. It is known that cysteine residues involved in the binding to ubiquitin, which is essential to the expression of E2 activity, are present in the UBC domain. To clarify the mechanism by which Ubc3 confers methylmercury resistance on yeast cells, we produced yeast cells overexpressing a mutant Ubc3 by substituting this cysteine residue with alanine, and found that these yeast cells were not methylmercury-resistant. Therefore, the ubiquitin transfer activity of Ubc3 is considered essential to the acquisition of methylmercury resistance.

Thirteen enzymes of the E2 family of yeast have been identified, each considered to exihibit substrate specificity. Therefore, we produced yeast cells overexpressing Ubc2, Ubc4, Ubc5 or Ubc7 of the E2 family, and determined their methylmercury resistance. Methylmercury resistance was observed in the yeast cells overexpressing Ubc4, Ubc5 and Ubc7. Our study was the first to show that high-level expression of E2 family enzymes confers resistance to toxic chemicals. Among the yeast cells overexpressing E1 (Uba1) or E3 (CDC53, SKP1, HRT1) proteins, only the yeast cells overexpressing Uba1 of the E1 family exhibited weak methylmercury resistance. These results suggest that E2 is the rate-limiting enzyme in the ubiquitination reaction, and the amount of ubiquitinated protein within cells was markedly increased by the overexpression of Ubc3.

It is considered that protein denatured by active oxygen is ubiquitinated by the ubiquitin system and degraded. It is hypothesized that cytotoxicity results when abnormal protein do not undergo normal degradation and accumulate in cells. Therefore, it is suggested that some modification of a specific protein within cells by methylmercury causes cytotoxicity, and enhancement of the degradation of the protein by ubiquitination reduces this toxicity. In this case, the ubiquitin system acts to protect cells against methylmercury toxicity, and the protein ubiquitinated following modification by methylmercury would be the target molecule of methylmercury toxicity.

There are genes in humans that are homologous to the genes encording GFAT and Ubc3, which have been identified as proteins conferring methylmercury resistance on yeast cells. Therefore, both proteins are likely to be involved in methylmercury toxicity in human cells. To clarify the mechanism of the toxicities of other toxic chemicals, investigation of resistance factors at the genetic level using yeast is considered useful.

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[F-1]

SAFETY MARGIN AND RISK PERCEPTION IS INSUFFICIENT ON EXPOSURE TO METHYLMERCURY AMONG JAPANESE CONSUMERS

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National Institute for Minamata Disease, Japan

Fish is one of important nutrient sources in Japan, and Japanese food habit is strongly dependent on fish and other marine products. It has been known that general exposure level to methylmercury is relatively high in Japanese population because fish and marine product is primary sources of methylmercury exposure (National Research Council 2000). A series of attempts was carried out to promote risk communication approach for the prevention of health effects by low-level methylmercury exposure through daily fish consumption. Attitude surveys were conducted on participants of the hair mercury analysis service counter that was set up in the two event sites of an environment problem exhibition "*Minamata Exhibition*" held in Kawasaki and Sapporo, Japan by a non profitable organization "MINAMATA Forum" (MINAMATA Forum 2001). Hair samples were collected with answer sheets of first questionnaire on 685 participants in the Kawasaki exhibition from January 6 through February 8, and on 1068 participants in the Sapporo exhibition from May 30 through June 13, 2004 (Fig.1). The first questionnaire contained food frequency questionnaire (FFQ) and questions concerning recognition on the fish consumption advisories that had been announced by the Ministry of Health, Labour and Welfare (MHLW) in June 2003 (Table 1). The participants received the analytical result on total mercury concentration of the hair sample with essential explanations for understanding of the data and were further requested to answer the second questionnaire that was used for the examination of attitudes after the notification. The determination of total hair mercury was described previously (Yasutake et al., 2003).

Fig.2 indicates observed distribution of hair mercury concentrations. The geometric mean of hair mercury concentration (mean years of age) of female and male participants was 2.80 μg/g (40.3 yrs) and 3.30 μg/g (37.8 yrs) in Kawasaki, and 1.71 μg/g (40.0 yrs) and 2.17 μg/g (37.0 yrs) in Sapporo, respectively. The average level was significantly higher in the Kawasaki exhibition than not only in the Sapporo exhibition but the average obtained in a more extensive survey on 14 districts in Japan with geometric mean of 1.65 μg/g and 2.47 μg/g, respectively (Yasutake 2005). Fish consumption was closely associated with the hair mercury level. Furthermore, the tendency of tuna preference as a frequently ingested fish was apparently correlated with hair mercury level and the relatively high mercury

concentration in the Kawasaki exhibition was consistent with high consumption of tuna in this district (The Ministry of Internal Affairs and Communications 2003). A provisional safety standard of methylmercury of MHLW is 170 μg/capita/week (average body weight is 50 kg) and corresponds to a hair mercury level of c.a. 5 μg/g. The calculation of cumulative frequency of hair mercury concentration (Fig.3) suggested that 5.8% and 1.0% of the participants of childbearing aged females (CBF) were exposed to methylmercury at higher level than the Japanese safety standard in Kawasaki and Sapporo, respectively. Furthermore, 62.5% and 29.5%, respectively, of CBF exceeded 2.2 μg/g, a hair mercury concentration that corresponds to another provisional tolerable weekly intake that was recently revised by Joint FAO/WHO Expert Committee on Food Additives in 2003.

Among participants of 20 years of age or older, only 47.8% (57.8% in Kawasaki and 41.4% in Sapporo) knew the announcement of MHLW advisories on the intake limitation of some whales, shark, swordfish and alfonsino for pregnant and may be pregnant women. In the participants who knew the announcement, 67.6% felt question or anxiety about the recommendation is limited for pregnant and may be pregnant woman and only a part of them (14.8%) accepted that the advisories are primary targeted for the specific sub-population. The frequency was significantly ($p<0.01$) lower in Kawasaki (11.2%) than in Sapporo (17.2%) where the information had been provided for effective understanding about methylmercury toxicity with some improvements after experience in Kawasaki including the addition of explanation on effective excretion of the chemical from human body. Among the female participants who knew the advisories, 27.4% made any restraint on fish intakes after the announcement. The frequency was significantly higher than in the males (11.2%). The tendency of restraint of fish intakes was slightly, but not significantly, prominent in females of fifty yrs of age or older (31.6%) than in CBF (24.8%). Indeed, the frequency of the fish restraint was the highest in 60s (43.9%) and the lowest in 20s (10.9%) among different age groups of female participants.

A large part of the participants, 57.4% had higher hair mercury level than they had expected before the analysis. Most of the participants (95.0%) thought that it was helpful to know own hair mercury level. About one third of the participants (36.3%) thought that the Japanese safety intake limit of methylmercury should be lowered. However, 37.5% reserved their answer to this issue. A frequency was 38.5% on the participants who felt any anxiety about their own hair mercury level. The frequency of anxiety was higher in females than males, but similar between females with ages younger than 50 yrs and elder ages. The anxiety frequency was analyzed in relation to the mercury concentration. No apparent correlation was observed between the hair mercury level and the frequency of female participants who worried about own mercury level, instead, the population average of hair mercury concentration appeared to be a criterion in the subjective impression of the results even though written information on several criteria levels had been provided including the domestic and international safety standard limits. The tendency was similar in CBF and

females of senior ages.

It was confirmed that the mercury content in hair of residents of metropolitan area of Japan was significantly higher than an average level in Japan. The consumption of tuna has been known to be relatively high in this region and it was also demonstrated that the tuna consumption is one of major determinants of methylmercury exposure in Japanese population. On the other hand, Japanese consumers' recognition is not enough, qualitatively and quantitatively, concerning exposure to methylmercury through ordinary fish intakes even though the population has relatively small safety margin on public exposure to the chemical. Supplying of necessary information is essential but not enough in risk communication. It is also important to help decision making of individual consumers and to support behavior alteration, if necessary. Hair mercury notification with sufficient information supplying system may be effective in promoting risk communication and to assist consumers' decision making for the risk avoidance. To protect Japanese population from possible health effects of low level methylmercury, it is not enough to reduce the safety exposure limit and make a strict standard of methylmercury. Instead, it may be more important, before the revision of the standard, to construct effective system to assist consumers such as child bearing aged females and young children to control their own methylmercury exposure level by improving fish consumption pattern.

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Table 1 Abstract of the guideline of fish intake that has been announced by the Ministry of Health, Labour and Welfare, Japan (a tentative translation)

Matters to be attended for pregnant and may be pregnant woman on intake of mercury containing marine foods

(abstract)

the Ministry of Health, Labour and Welfare, Japan

June 3, 2003

Since fish contains trace amounts of methylmercury, it is desirable for pregnant and may be pregnant woman to heed following instructions on intake of fish and marine food to prevent effects of methylmercury on fetus.

Bottlenose dolphin (60-80g per serving)	not more than once in two months
Baird's beaked whale, Short-finned pilot whale, Sperm whale, Shark (muscle) (60-80g per serving)	not more than once in a week
Sword fish, Alfonsino (60-80g per serving)	not more than twice in a week

Fish intake is generally beneficial for human health and these instructions are hoped to be understood adequately and not to cause decline of fish intakes.

Fig 1 Posters of Kawasaki and Sapporo Exhibitions, and frequency of resident city among participants of hair mercury analysis service

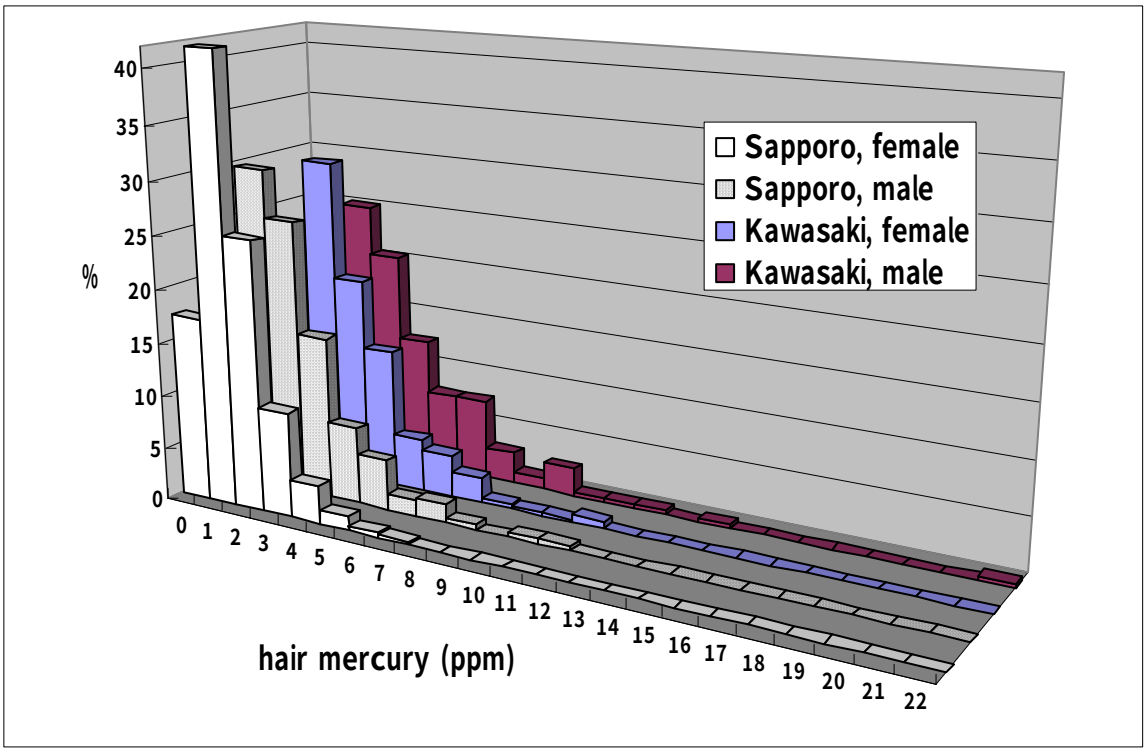

Fig. 2 Distribution of hair mercury concentrations.

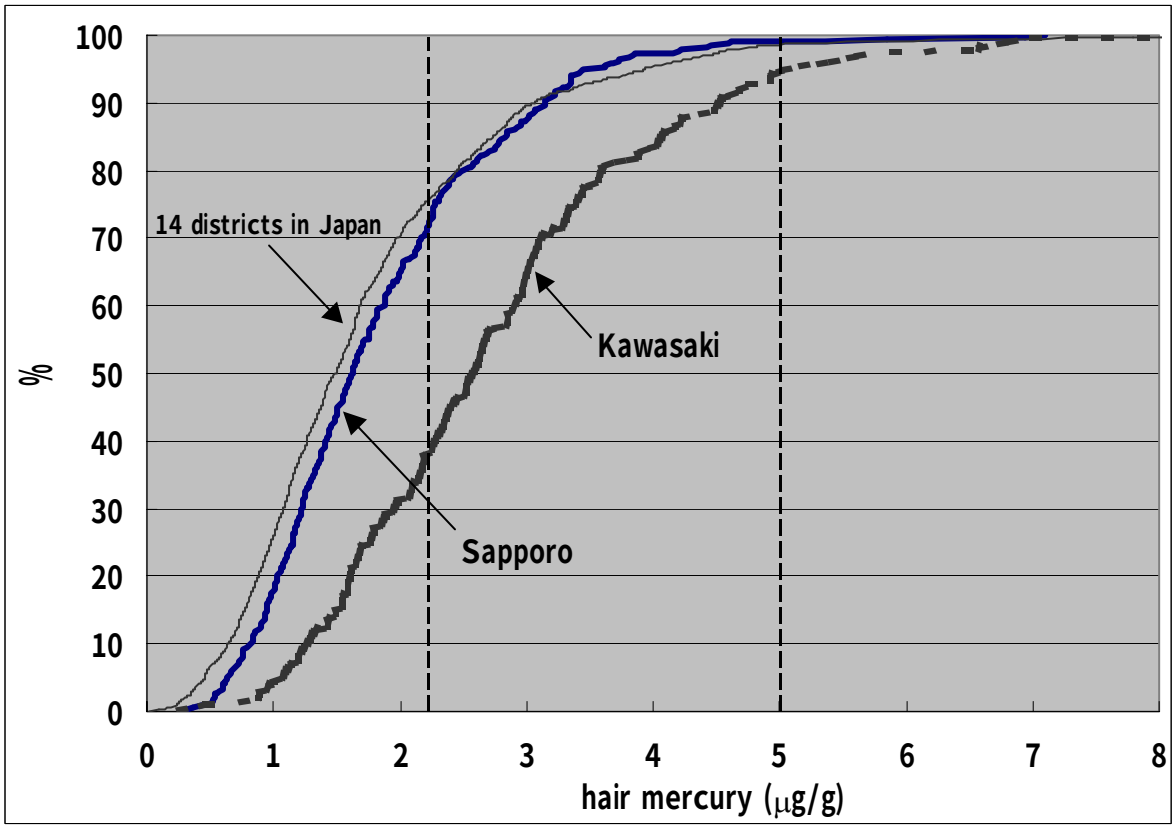

Fig.3 Cumulative frequency of hair mercury concentrations among child bearing aged females

[F-2]

OMEGA-3 FATTY ACIDS AND CONTAMINANTS IN FISH: HOW DO WE BALANCE RISK AND BENEFIT?

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Current Problems in Risk Evaluation and Risk Management of Methylmercury and Cadmium, forum sponsored by the National Institute for Minamata Disease, Minamata, Japan, 2005

Uncontaminated fish is a healthy food. It is a good source of protein, and is low in saturated fat. It also provides the most concentrated source of omega-3 polyunsaturated fatty acids (PUFAs): specifically decosahexaenoic acid (DHA). Increased body burdens or intakes of DHA were associated in some studies with decreased hyperglycemia, hypertension, and cardiac death. *In utero* or infant exposure to DHA has also been reported to be associated with improved neuropsychological function.

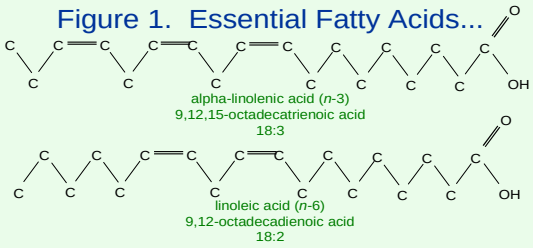
Metabolism of fatty acids

DHA is not an essential fatty acid in humans. It can be manufactured from the essential fatty acid (FA) α-linolenic acid by a series of desaturation and elongation steps (Figure 1). The other essential fatty acid, linoleic acid, is an omega-6 fatty acid, and is converted to arachidonic acid via the same enzymes used for conversion of α-linolenic acid to DHA (Figure 2). Therefore there is competition for conversion of the essential omega-3 or omega-6 fatty acids to their long-chain catabolites. The omega-3 and omega-6 PUFAs are constituents of cell membranes, as well as being precursors to hormones and other signaling molecules.

The main source of linoleic acid in the American diet is corn oil. Other nut oils are also sources of linolenic acid, as well as meat from grain-fed animals. The richest source of α-linolenic acid is flax seed, which is not consumed routinely, at least by Americans. Other sources include some nut oils, dark green leafy vegetables (in low concentrations) and meat from grass-fed animals, including wild game. The natural ratio of omega-6 to omega-3 in the diet is between 2:1 and 5:1, based on estimates of prehistoric diets as well as modern gatherer/hunter societies. The ratio in the Japanese diet was traditionally about 4:1 (Sugano,

1996), although the Japanese diet is beginning to be more Western (Umemura *et al*., 1993). In contrast, the ratio of omega-6 to omega-3 in the American diet is as high as 20:1, presumably as a result of a diet that is based on corn and corn-fed livestock.

Figure 1. Essential Fatty Acids...



and Long-Chain *n*-3 Fatty Acids

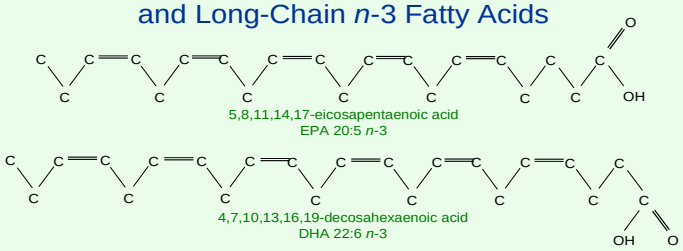
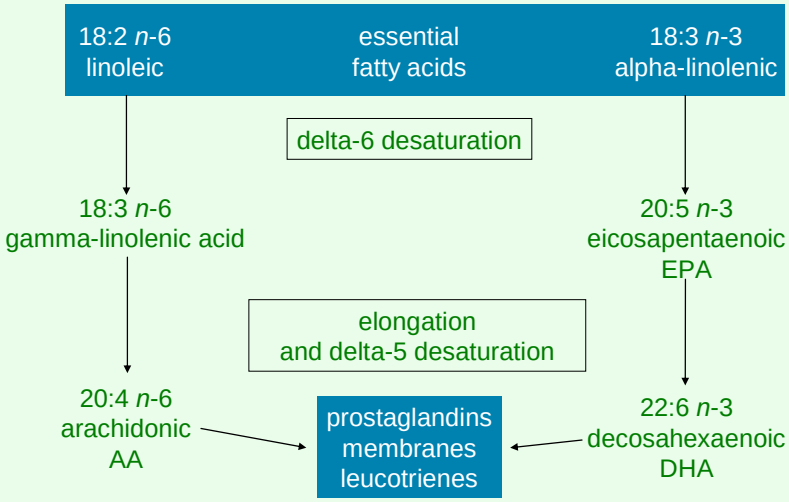


Figure 2. Simplified Scheme for Conversion of Essential Fatty Acids to Their Long-Chain Metabolites



DHA is actively transported across the placenta, as is the essential fatty acid linolenic acid (see Lauritzen *et al*., 2001 for review). The adult, fetal, and term and pre-term infant liver can synthesize both omega-3 and omega-6 PUFAs. Breast-fed infants typically have higher body burdens of DHA than formula-fed infants. Until recently (and in many formulations currently), infant formula did not contain long-chain PUFAs or essential FAs. The primary determinant of PUFA composition in maternal milk are maternal diet, adipose stores (historical diet), and hepatic conversion. It is estimated that about 30% of long-chain PUFAs in breast milk comes directly from current diet. There is in most studies a correlation between long-chain PUFAs in the mother and the fetus.

FA requirements in the developing brain

DHA is an important constituent of cell membranes in the nervous system. The outer rod segment of the retina is particularly rich in DHA, with DHA comprising 50% of total esterified fatty acids. Manipulation of DHA levels in *in vitro* visual systems affects the functionality of those systems (Lamb, 1996), and transgenerational deficiency produced by dietary manipulation produces functional visual deficits in both rodents and monkeys (Wheeler *et al*., 1975; Neuringer *et al*., 1984). DHA-containing phospholipids are also concentrated in synaptic membranes, and correlate with development of synapses (Brown, 1994; Neuringer, 1993). DHA deficiency also affects dopamine regulation in frontal cortex (Delion *et al*., 1994), a system that is important for cognition and organization of behavior. In fact, deficiencies in long-chain PUFAs results in cognitive deficits in rats and monkeys (Lamptey and Walker, 1976; Wainwright *et al*., 1999; Sheaff Greiner *et al*., 1999).

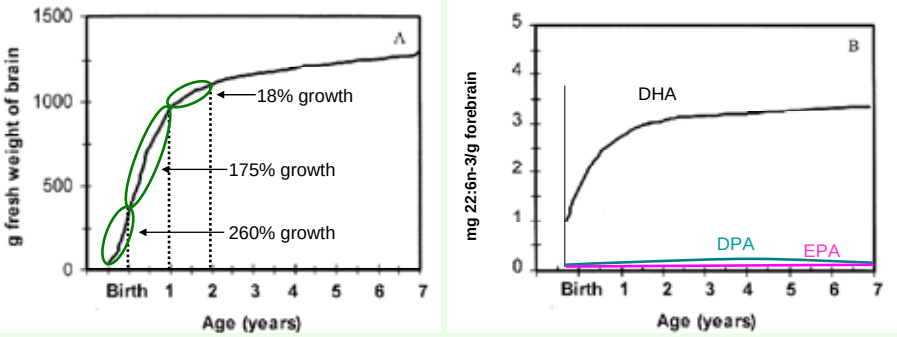
The fetus and infant have a substantial requirement for long-chain PUFAs for normal development of the nervous system. The brain grows 175% during the first year of life, continues to grow rapidly until about 18 months of age, and grows more slowly thereafter (Figure 3). Over 80% of the long-chain PUFAs in the central nervous system in humans are incorporated during the last trimester of pregnancy.

Evidence for beneficial effects of long-chain PUFAs during development on neuropsychological function

There are a number of studies on the effects of DHA supplementation to preterm infants on cognitive and visual function (Faldella *et al*., 1996; Uauy *et al*., 1990; Birch *et al*., 1992; Carlson *et al*., 1991, 1993, 1994, 1996a; Werkman and Carlson, 1996; Carlson and Werkman, 1996). These studies have almost universally found a beneficial effect of DHA supplementation. This is not surprising, given that the control formula contained not only no DHA, but no precursor(s) from which to synthesize DHA or other long-chain PUFAs. Perhaps more surprising is the contradictory results obtained in studies with full-term infants. Both positive and negative results have been observed for both cognitive and visual function (Carlson *et al*., 1996b; Auestad *et al*., 1997; Jørgensen *et al*., 1998; Makrides *et al*., 1995,

2000; Birch *et al*., 1998, 2000; Scott *et al*., 1998; Lucas *et al*., 1999; Agostoni *et al*., 1995; Willatts *et al*., 1998). It has been hypothesized that a minimum level of DHA in formula is required for beneficial effects to be observed (Lauritzen *et al*., 2001).

Figure 3. Human Brain Growth Spurt and DHA Accretion



Martinez, Brain Res., 1992

The results of studies of supplementation of infants are interesting and informative; however, the central question is whether increased long-chain PUFA intake by women results in improved neuropsychological performance in the offspring. There are a number of epidemiological studies that have addressed this issue. In a study in 83 breast-fed infants in British Columbia, Canada, performance was assessed on a number of tasks (Innis *et al*., 2001). Infants were divided into tertiles based on DHA levels in the infants' red blood cell-phosphotgalyl choline (RBC-PE) levels. Infant DHA status was associated with better visual acuity at 2 and 12 months, but not at 4 and 6 months. DHA levels were associated with better speech sound recognition at 9 months, but had no effect on visuospatial ability, Bayley Scales at 6 and 12 months, or novelty preference (short-term memory).

Visual acuity was assessed using visual evoked potentials in 39 four-month-old infants in Denmark (Jørgensen *et al*., 2001). Better acuity was associated with maternal fish intake and breast milk DHA levels.

The association between maternal fish diet, maternal RBC-PE levels, and vision was examined in a study in the United Kingdom of 435 children (Williams *et al*., 2001). Maternal RBC-PE levels were associated with moderate intake of oily fish (dichotomized as once in two weeks vs. more than once in two weeks). Breast feeding was the best predictor of depth perception at 3.5 years, then ingestion of oily fish. DHA levels in maternal RBC-PE were a weak predictor of visual function, but DHA levels were only available from 150 mothers.

In a study from the same cohort of children (Daniels *et al*., 2004), cognitive development in about 5000 children was assessed using the MacArthur Communicative Development Inventory (MCDI) at 15 months of age and the Denver Developmental Screening Test (DDST) at 18 months of age. Both tests are parent-completed assessments, and were mailed to the mothers. Intake of foods other than fish was not included in the analysis, nor were e.g. total protein levels. Better performance on both tests was associated with maternal and infant intake of fish, either fatty fish or white fish. PUFA levels were not measured. The results suggest that effects were the result of a constituent of fish other than omega-3 fatty acids. However, the dietary questionnaire did not adequately differentiate fish containing high levels of omega-3 fatty acids; the way in which respondents dichotomized "oily" versus "white" fish is unknown. DHA levels were not reported in this study.

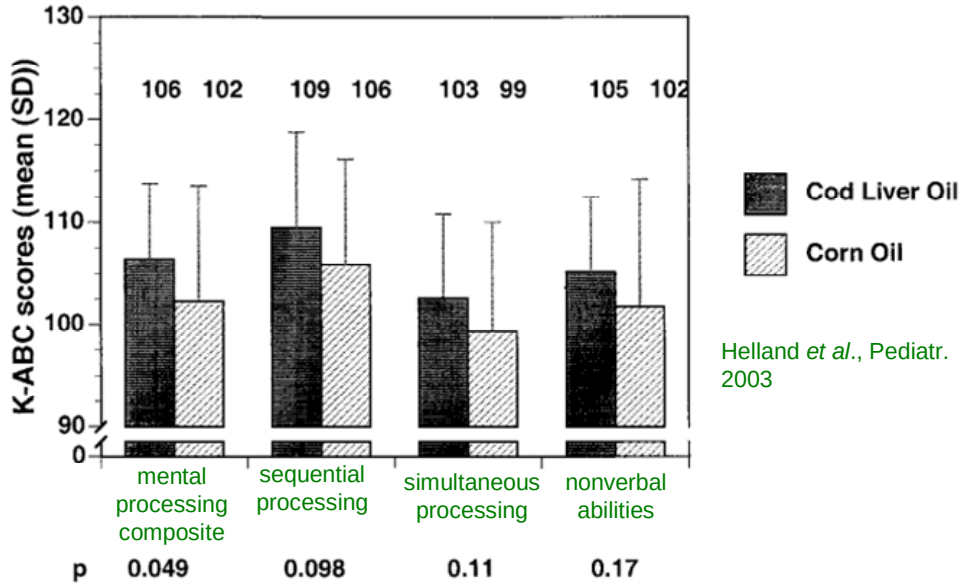
In a study of 128 four-year-olds in the Netherlands (Ghys *et al*., 2002), DHA and arachidonic acid (AA) levels were measured in umbilical plasma and RBC-PE. There were no associations with performance on the Groeningen Developmental Scale. This tests included items of the motor scale of the McCarthy Scales of Children's Mental Abilities as well as the Kaufman Assessment Battery for Children (K-ABC). The expected associations were observed with maternal IQ, breast-feeding, paternal education, and smoking during pregnancy, suggesting that the study had sufficient power. However, no DHA or AA concentrations were presented, so it is difficult to interpret the results.

A study in Sweden examined the effects of fatty acids on IQ at 6.5 years in 73 children (Gustafsson *et al*., 2004). Fatty acids were measured in colostrum and in breast milk at 1 and 3 months; fatty acids in blood were measured in umbilical cord and at 3 and 18 months. Breast-feeding was associated with higher IQ on the WISC III. There was an association between IQ and DHA levels in colostrum only, but not any other tissue (blood or milk). There was no association with other fatty acids. Although maternal education was measured, maternal IQ was not.

In a study of about 100 mother-infant pairs in Norway, women were supplemented with 10 mg/day of corn oil or cod liver oil from the second trimester of pregnancy to three months postnatally (Helland *et al*., 2001, 2003). This provided 1.18 gm of DHA, 0.80 mg of EPA, and 2.49 mg of total omega-3 PUFA a day. The cod liver oil group had higher levels of DHA in umbilical cord phospholipids. There was no effect of DHA supplementation on preferential looking at 6 and 9 months, despite the high intake of DHA. At four years, 84 children were assessed on the K-ABC. The cod liver oil group performed better on one scale of the K-ABC but not others (Figure 4); this scale was associated with DHA levels at 4 weeks but not birth or three months. The minimal effect, despite the high level of DHA intake, suggests that the effect of DHA, if there is one, is small. In addition, corn oil supplementation to the reference group may have prevented maternal conversion to long-chain omega-3 PUFAs because of competition for enzymes in the omega-6 pathway. Third, the observed effect may result from some other constituent of cod liver oil, since only 10% of the mixture

was DHA. Last, maternal IQ was not measured. This may be less important in this randomized study than in the epidemiological studies described above, but is nonetheless an important flaw in the study.

Figure 4. IQ Scores at 4 Years in Children Whose Mothers Took Corn or Cod Liver Oil



Helland *et al*., Pediatr. 2003

Levels of DHA in milk in human populations: How much is necessary?

Average levels of DHA in maternal milk in various countries and studies vary considerably (Table 1). The highest levels are observed in Japan, who consume high amounts of fish and marine mammals. In contrast, levels in the U.S. population are among the lowest in the world, with suboptimal ratios of omega-6 to omega-3.

If one assumes that the effects observed in the Helland *et al*. study are a consequence of DHA supplementation, the body burdens of FAs in this study can be compared to those typically observed in various populations. The average DHA level in the supplemented group was 1.4%, higher than the average Japanese level and about the same as that of Inuit populations (Innis and Kuhnlein, 1988) (Table 2). The average DHA level of the corn oil group was 0.42%, which is higher than typical levels in the U.S. population. An important gap in our understanding is the dose-effect function for beneficial effects. Is there a minimum effective level below which there are no benefits? Is there an asymptote for benefits on neuropsychological function, above which there are no further benefits? And what is the shape of the curve? Until these important issues are understood, it is impossible to make informed choices concerning risk versus benefit of seafood intakes.

Table 1. Relative Content of PUFAs in Human Milk

Country	*n*	22:6*n*-3	*n*-6/*n*-3	Country	*n*	22:6*n*-3	*n*-6/*n*-3
Japan	562	1.00	4.7	France	24	0.24	9.1
Japan	53	0.53	6.0	France	15	0.14	12.0
Norway	22	0.38	7.6	Italy	18	0.3	9.6
Denmark	17	0.43	5.9	Spain	28	0.4	8.9
Denmark	25	0.38	6.7	Spain	40	0.35	9.1
Denmark	17	0.53	4.9	Australia	61	0.32	9.5
Denmark	39	0.35	5.6	Australia	23	0.26	10.4
Sweden	17	0.24	5.8	Canada	17	0.2	7.4
Finland	16	0.18	7.1	U.S.A.	77	0.06	9.9
Germany	15	0.22	9.0	U.S.A.	30	0.16	12.9
Netherlands	99	0.19	10.0	U.S.A.	33	0.16	27.8
England	21	0.37	13.5	U.S.A.	10	0.29	9.8
France	32	0.37	9.7	U.S.A.	14	0.2	17.8

adapted from Lauritzen *et al*., 2001

Table 2. Breast Milk Composition in Norwegian Study Versus Levels in Japan and U.S.

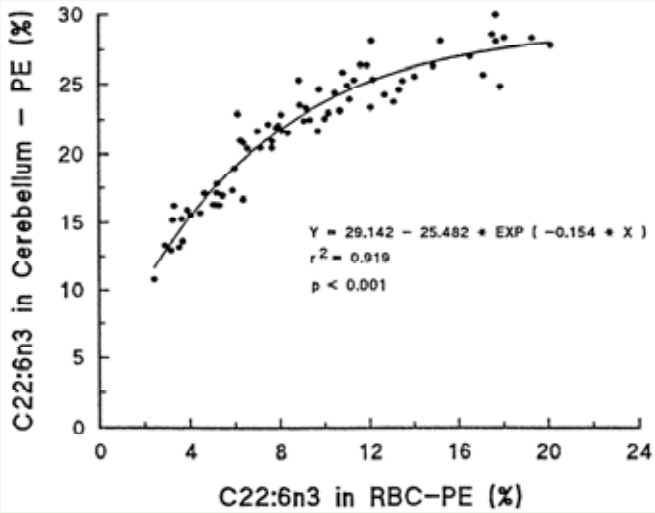
	fatty acids (wt %)				
	18:3 *n*-3	20:5 *n*-3	22:6 *n*-3	20:4 *n*-6	*n*-6/*n*-3
Norway					
corn oil	0.85	0.12	0.43	0.42	8.3
cod liver oil	0.92	0.42	1.41	0.38	2.0

There is some information that informs these issues. In studies with preterm infants, the lowest concentration of DHA supplementation, used in several studies, was 0.2%. This level was sufficient to produce beneficial effects. In studies with term infants, on the other hand, the lowest effective level may be 0.30-0.35%, based on results of multiple studies (Lauritzen *et al*., 2001). It is important to remember, however, that human breast milk has a different composition than commercial formula, so that extrapolation from formula studies may not be straightforward.

It can be assumed that there is an asymptote above which there is no further gain produced by increased DHA levels. This is based at least two asymptotic relationships: first, there is an asymptotic relationship between DHA levels in blood and those in brain (Figure 5); second, there is an asymptotic relationship between the DHA in the RBC-PE of the mother

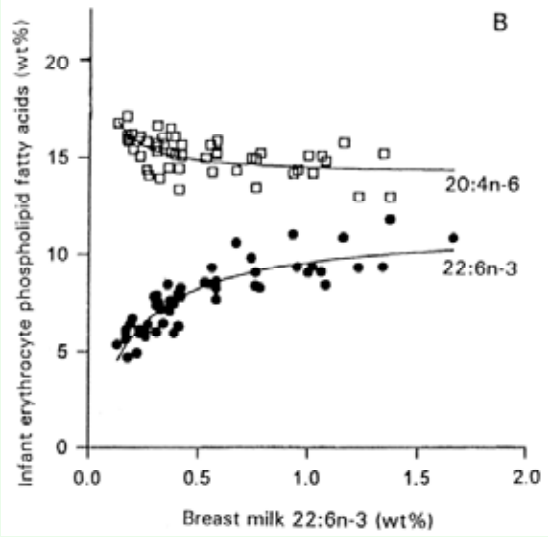
and the concentration transported into breast milk (Figure 6). It is in fact obvious that at some point further increases in DHA intake will not result in increased DHA levels being incorporated into brain. Unfortunately, it is unknown what that intake level is.

Figure 5. Relationship Between DHA in Rat Cerebellum and Red Blood Cells



Ward *et al*., J. Nutrit. 1998

Figure 6. Relationship Between Human Erythrocyte DHA and Arachadonic Acid and DHA in Breast Milk



Gibson *et al*., Eur. J. Clin. Nutr., 1997

Risk assessment for neuropsychological damage produced by* in utero *exposure to methylmercury

The U.S. Environmental Protection Agency reference dose (RfD) for methylmercury (Rice *et al*., 2003) is based on a report by the U.S. National Research Council National Academy of Science (NRC, 2000). The NRC committee performed a benchmark dose (BMD) analysis of three longitudinal prospective studies, two of which found adverse effects and one of which did not. The BMD was based on a doubling of the number of children in the 5th percentile of performance on a number of endpoints. The EPA then used a one-compartment model to estimate the mercury intake of the mother associated with the BMDL (lower limit confidence interval on the BMD). EPA used central tendencies in the model, rather than distributions of the relevant parameters. In addition, EPA assumed a methylmercury cord:maternal blood ratio of 1.0, even though it was recognized that the assumption was inaccurate. EPA then applied a total uncertainty factor (UF) of 10, considering a factor of 3 to be for pharmacokinetics (PK) and an additional 3 for pharmacodynamics. The PK factor was considered to be data-derived, since published kinetic studies documented 2-3 times variability between women for elimination half-life. There was no factor included for the fact that the point of departure was a defined effect level rather than a no-effect level.

EPA recognized that a parametric analysis of the PK model would contribute significantly to the understanding of inter-individual variability. A distributional analysis was performed by Dr. Alan Stern (Stern, 2005). He chose 58 ug/L mercury in cord blood as the BMD, as recommended by the NRC, and used a one-compartment PK model. A distributional analysis for the ratio of cord:maternal blood had been performed previously (Stern and Smith, 2003). The model outputs of the full distributional analysis are provided in Table 3. Variables included in the analyses, for which data were obtained from studies in appropriate populations, were maternal elimination half-life, maternal blood volume, maternal body weight, and fraction of dose absorbed and fraction of absorbed dose in blood. (The latter two parameters contributed to little variability.) The mean maternal intake associated with a cord blood concentration of 58 ug/L was 0.99 ug/kg/day, similar to the 1.1 ug/kg/day derived by EPA. However, the 50th percentile was 0.81 ug/kg/day, lower than the mean. The 5th percentile was 0.30 ug/kg/day, and the 1st percentile was 0.20 ug/kg/day. The ratio of the 50th/5th percentile was 2.7, and the 50th/1st percentile was 4.0. Therefore a factor of 3 for PK variability is substantiated to protect 95% of the population of pregnant women from a blood mercury level of 58 ug/L; a factor of 4 is required to protect 99% of pregnant women. It must be remembered that 58 ug/L is an *effect* dose; further, there is no evidence for a threshold for adverse effects from methylmercury in the data from the studies modeled by the NRC.

Acceptable daily intakes of PCB and dioxin TEQ

A number of agencies have derived acceptable daily intakes for TEQ. The World Health Organization (WHO) developed a range of 1-4 pg/kg/day. The UK Committee on Toxicology considers 2.0 pg/kg/day an acceptable intake, whereas the U.S. Agency for Toxic Substances and Disease Registry considers 1.0 pg/kg/day an acceptable level. The State of Maine uses 1.0 pg/kg/day in risk management decisions.

Table 3. Model Output for Maternal Intake Corresponding to 58 ug/L Cord Blood

	maternal intake (ug/kg/day)
mean	0.99
1st percentile	0.20
5th percentile	0.30
10th percentile	0.37
50th percentile	0.81
50th/5th percentile	2.7
50th/1st percentile	4.0

Alan Stern, EHP, online

Table 4. Grams of Various Fish Species Required for Maternal Ingestion of 1.0 gm of DHA/day

	DHA (g/100g)	gm/day to get 1 gm DHA
fresh salmon (Atlantic, chinook)	1.3	77
mackerel	1.1	91
fresh tuna (max)	1.1	91
sardines	0.7	143
herring	0.6	150
fresh trout	0.3	333
halibut	0.3	333
swordfish	0.2	500
canned light tuna	0.2	500

Table 5. Contaminants in Fatty Fish

	Hg (ug/g)	dioxin & PCB TEQ (ng/kg fresh weight)
salmon		
Europe	0.05	3.0
Chile	0.05	1.0
Alaska wild	0.05	0.2
mackerel (not king)	0.20	3.1
fresh tuna	0.30	0.7
sardines	0.03	????
herring	0.05	8.6
trout	0.10	1.3
halibut	0.30	
swordfish	1.0	
canned light tuna	0.10	

Table 6. Maternal Ingestion of Contaminants Associated With 1.0 g/day Intake of DHA

	Hg ug/day / ug/kg/day	pg TEQ/day / pg/kg/day*
salmon		
Europe	3.85 / 0.06	256 / 4.2
Chile	3.85 / 0.06	85 / 1.0
Alaska wild	3.85 / 0.06	15.4 / 0.26
mackerel (not king)	18.2 / 0.30	282 / 4.7
fresh tuna	27.3 / 0.45	63.7 / 1.1
sardines	4.5 / 0.07	????
herring	7.5 / 0.12	1290 / 21.5
trout	33.3 / 0.55	256 / 4.3
halibut	99.9 / 1.7	
swordfish	500 / 8.3	
canned light tuna	50 / 0.83	

* based on 60 kg woman

Comparison of beneficial DHA intake from intake of fish and associated intake of contaminants

Based on the Helland *et al*. (2003, 2001) study, intake of 1.0 gm of DHA has small beneficial effects for cognitive development. There are data available from various sources in the DHA levels in various fish species that are high in omega-3 FAs (Table 4). There is

variability in the data from various sources; nonetheless, an estimate may be made of the amount of fish of various species that would have to be consumed per day to receive 1.0 gm of DHA. For species high in DHA (salmon, mackerel, tuna), the amounts are reasonable, less than 100 gm/day. However, the amount of some species that would need to be consumed is very high for some species such as swordfish, and would be very unlikely to be consumed in the U.S. population.

Unfortunately, most of these species contain significant concentrations of methylmercury and/or TEQ (Table 5). Salmon, sardines, and herring are low in methylmercury, whereas swordfish, fresh tuna, and halibut are high. However, herring (at least Atlantic herring) is very high in TEQ. The TEQ in salmon is highly dependent on the source, with European salmon purchased in the grocery store having high TEQ levels, and Pacific wild salmon having relatively low levels.

These data can be used to estimated the amount of methylmercury and TEQ a woman would ingest from various fish species if she ate enough of any one species to ingest 1.0 gm of DHA (Table 6). Depending on the value chosen as an acceptable daily intake, a number of species do not constitute an appreciable risk based on methylmercury tissue levels. Based on either EPA RfD of 0.1 ug/kg/day or the WHO level of 0.2 ug/kg/day, salmon, sardines, and herring may be eaten. However, herring is very high in TEQ, and a TEQ for sardines could not be identified. If an acceptable TEQ of 2.0 pg/kg/day is used, either Chilean or Alaskan salmon is acceptable. Fresh tuna is as well, but it is high in methylmercury. If a level of 1.0 pg/kg/day is used as an acceptable daily intake, only consumption of wild Alaska salmon is acceptable for pregnant women. Chilean salmon would provide the entire TEQ, allowing no margin for consumption of other foods containing TEQ.

Conclusions for balancing risk and benefit

The evidence for the beneficial effects of ingestion or body burden of DHA in pregnant women on neuropsychological function in offspring is weak. Most of these studies contain serious methodological flaws. Maternal IQ is the most important single determinant of the child's IQ. Maternal IQ is also likely to be associated with dietary habits, such as increased fish intake, that would result in increased DHA in mother and infant. It is therefore a serious flaw that only one study (Ghys *et al*., 2002) measured maternal IQ. This is also the only study that found no association between DHA and cognitive development. In addition, only one study (Daniels *et al*., 2004) measured the HOME score, an important measure of the child's access to cognitive stimulation. The lack of control for maternal IQ and a measure of the home environment represents lack of control of important potential confounding variables that may be responsible for the reported effects. Other important variables, such as smoking or alcohol consumption during pregnancy, are not included or are included as dichotomous (yes/no) variables rather than continuous variables. This represents suboptimal covariate control. In addition, observed effects were often associated with some measures of DHA (or

fish intake) and not others, and in some studies DHA levels were associated with some measures but not others. This suggests that if there is an effect, it is a weak one.

In contrast, the evidence for adverse effects of the contaminants methylmercury, dioxins and PCBs is considerably stronger, and relies on multiple studies in relatively large cohorts. In addition, covariate control, including measurement of maternal IQ and home environment, was significantly better in the contaminant studies.

Even assuming that a 1.0 mg intake of DHA in the Helland *et al*. study (2003, 2001) is beneficial, ingestion of an unrealistic amount of fish of most species is required to meet that requirement. In addition, ingestion of most species in the required amount would result in an unacceptable intake of methylmercury and/or TEQ.

On an even more fundamental level, the requirement for dietary long-chain PUFAs versus the essential fatty acids is unclear. In studies in animals, supplementation with α-linoleic acid resulted in the same FA composition in brain and retina as supplementation with DHA (Craig Schmidt *et al*., 1996; Wainwright *et al*., 1999; Ward *et al*., 1998; Arbuckle *et al*., 1994). For example, in a study in piglets, formula supplemented with 4% α-linolenic acid resulted in as much DHA in brain and retina as did mother's milk (Arbuckle *et al*., 1994). This suggests that the piglets were capable of synthesizing sufficient DHA if provided with sufficient amounts of the essential fatty acid precursor. In addition, transgenerational deficiency of α-linolenic acid produces deficits in behavior in animals (Okaniwa *et al*., 1996; Sheaff Greiner *et al*., 1999). It is unclear the degree to which supplementation of pregnant women with α-linolenic acid would result in increased breast milk levels of either α-linolenic acid or long-chain PUFAs as a consequence of endogenous synthesis. The experiment has apparently not been performed. It may be that the conflict between the benefits of fish versus contaminants in fish is a false one. If supplementation of the essential fatty acid results in sufficient long-chain PUFAs in the fetal nervous system, putting the fetus at risk for contaminant exposure through maternal intake of contaminated fish is unnecessary.

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[F-3]

EFFECTS OF COMBINED EXPOSURE TO PCBS AND METHYL MERCURY ON COGNITIVE AND MOTOR FUNCTION

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Polychlorinated biphenyls (PCBs) and methyl mercury (MeHg) are widespread environmental contaminants that bioaccumulate in aquatic ecosystems. Consumption of fish from contaminated waters is a major source of exposure in humans. Nervous system effects have been reported in laboratory animals and humans following developmental exposure to PCBs or MeHg, but studies evaluating the effects of combined exposure to these two neurotoxic agents were lacking. In the research reported here, we evaluated the long-term effects of combined exposure to PCBs and methyl mercury during early development on cognitive and motor function in laboratory rats. Female Long Evans rats were exposed to PCBs alone (6 mg/kg/day Arocolor 1254 in corn oil), MeHg alone (0.5 ppm in the drinking water), both PCBs and MeHg or the corn oil vehicle only. The females were exposed daily for four weeks and then bred to unexposed males. Exposure of the females continued throughout gestation and the early postnatal period. PCB and MeHg exposure terminated on postnatal day 16 and pups were weaned on postnatal day 21.

In early adulthood one male and one female from each litter were evaluated on three tests of balance and coordination, a rope climbing task, parallel bars and a rotating rod task in which the animals were required to cross a two-meter long rotating rod to reach a platform. The most striking effects were observed on the rotating rod task. Pups exposed to either chemical alone did not differ significantly from control pups, but those exposed to both MeHg and PCBs were markedly impaired relative to unexposed controls, suggesting an additive effect of the two contaminants on motor function.

Another male and female from each litter were tested on a battery of cognitive tasks which included tests of cognitive flexibility and working memory. The working memory task, known as delayed spatial alternation, required the animals to alternate their responses between two levers in an operant testing chamber. Variable delays ranging from 0-18 seconds were interspersed between the trials in random order in order to assess working memory. Both contaminants produced significant reductions in the proportion correct, but exposure to the two chemicals in combination did not increase the magnitude of the deficit. Further analysis

revealed that reductions in proportion correct were present across all delays, suggesting a deficit in some aspect of learning or attention rather than working memory. Analysis of errors patterns across trials revealed an increase in perseverative errors in all three exposed groups.

In summary, the potential for developmental exposure to PCBs and MeHg to result in additive effects on cognitive and motor function was evaluated in laboratory rats. Combined exposure to the two chemicals did appear to have additive effects on balance and coordination as measured on a rotating rod task. In contrast, both chemicals caused impairments on a working memory task, but combined exposure did not result in a larger effect than was seen with either chemical alone. This research was supported by grant numbers ES05885 and ES011263 from NIEHS and R82939001 and R82895301 from the USEPA.

[F-4]
THE MANY FACES OF MERCURY

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The Agency for Toxic Substances and Disease Registry (ATSDR), a component of the U.S. Department of Health and Human Services, is charged by the U.S. Congress with developing Toxicological Profiles for the 275 substances most commonly found at hazardous waste sites in the United States. Mercury is the third most highly identified substances as such sites, and the current copy (March 1999) of the Toxicological Profile for Mercury represents the second update of that document.

The hazards of methylmercury ingestion have been well studied and reported. Poisoning episodes in Minamata, Japan in the 1950's and in Iraq in the 1960's and 1970's have profoundly demonstrated the frank toxic effects of inadvertent exposure to methylmercury. Both the Japan and Iraq poisonings, however, represent high dose exposures over a limited period of time. The overwhelming preponderance of data regarding chronic, low-level dietary intake from fish ingestion, on the other hand, come from well constructed prospective, longitudinal epidemiology studies in the Faroe and Seychelles Islands and a third excellent epidemiology study conducted in New Zealand. Based upon the data from all these studies, many governments have taken aggressive measures to mitigate or eliminate unnecessary exposures to methylmercury.

However, the exposure of humans to high levels of inorganic forms of mercury continues throughout the world. Gold mining, cultural/religious uses, oral and laboratory thermometers, barometers, small batteries, thermostats, light switches, dental amalgam fillings, some cosmetic products, and medicines, as well as choralkali and fluorescent light bulb manufacturing, all represent potential sources of exposure to inorganic forms of mercury.

This presentation will discuss ATSDR’s on-going efforts to keep abreast of emerging data on various forms of mercury as they become available, and the Agency’s efforts to mitigate unnecessary exposures to that heavy metal.

[F-5]

CHANGES IN METHYLMERCURY EXPOSURE TO OFFSPRING THROUGHOUT GESTATION AND SUCKLING: A MINI-REVIEW

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Abstract:

This paper provides a concise review of involving a description of our recent research results from animal and human studies. Higher methylmercury (MeHg) accumulation and susceptibility to toxicity in the fetus than in the mother at parturition is well known. However, the degree of MeHg accumulation in the brain during the late pregnancy period when the human brain is most vulnerable is not clear. In the human, the effects of MeHg exposure on pregnant and breast-feeding women remain an important issue for elucidation, especially those of continuous uptake in high-fish-consumption populations. The purpose of this study was to evaluate the changes in MeHg accumulation in offspring throughout gestation and lactation. In animal study, adult female rats were given a diet containing 5 μg/g Hg (as MeHg) for 8 weeks. Then they were mated and subsequently given the same diet throughout gestation and lactation. On embryonic days 18, 20, 22 and at parturition, the concentrations of Hg in the brains of offspring were approximately 1.5-2.0 times higher than those in the mothers. On the other hand, during the suckling period Hg concentrations in the brain rapidly declined to about 1/10 of that during late pregnancy. Changes in Hg accumulation in blood and liver after parturition were similar to those in the brain. In human study, Hg concentrations in red blood cells (RBCs-Hg) in 16 pairs of maternal and umbilical cord blood samples were compared at birth and 3 months of age after parturition. RBCs-Hg concentration in the umbilical cords was

higher than those in the mothers at parturition. The Hg concentration of RBCs-Hg in umbilical cords (13.0 ng/g) was about 1.6 times higher than that in the mothers (8.19 ng/g). There was a strong correlation in RBCs-Hg in mothers and umbilical cords. However, all the infants showed declines in Hg concentrations throughout the breast-feeding period. The Hg concentration in RBCs-Hg at 3 months of age was 6.87 ng/g, accounting for 53 % of that in the umbilical cords.

Both the animal and human studies indicated the MeHg exposure to the offspring might be especially high throughout the late gestation period but dramatically decreases during the suckling period. Therefore, intensive attention should be focused on the gestation rather than on the breast-feeding period to avoid the risk of MeHg to human infants.

Introduction

Fetuses and neonates are known to be high-risk groups for MeHg exposure.[1-3] In the epidemics in Minamata and Niigata, Japan, and in Iraq, many infants were congenitally affected by MeHg.[3-6] The clinical reports of patients in the Minamata district described the infant victims as having severe cerebral palsy, while their mothers had only mild manifestations of the poisoning, indicating a higher risk in fetuses.[5-7] The higher MeHg accumulation at parturition in the fetal brain than that in the mother is well established.[8-15]

Rapid brain growth occurs primarily during the third trimester in human[16] and the brain at the period is known to be most vulnerable to the toxicity of MeHg.[17] Takeuchi[6,7] suggested that MeHg affected the nerve cells after considerable differentiation had occurred during the intermediate or later period of fetal life. Therefore, MeHg accumulation during the late pregnancy deems to be most important to consider the adverse effects of MeHg to the developing brains. However, Hg accumulations in the brain throughout late pregnancy and changes in Hg accumulation in other tissues during the suckling period have not been well studied.

In the human, the effects of MeHg exposure on pregnant and breast-feeding women remain an important issue for elucidation, especially those of continuous uptake in high-fish-consumption populations.[3,18-21] MeHg can be transferred to offspring through breast milk, in addition to its passage through the placenta during intrauterine life.[4,18,21-23] Even though the Hg concentration in breast milk is known to be very low, [21,23-25] some recent studies suggested that infants reared on breast milk for a long period might be at increased risk.[26,27]

The purpose of this paper was to concisely review the MeHg exposure to offspring throughout gestation and suckling from our recent animal and human studies[23,28]. The number of the subjects in this human study increased from 7 of the previous study[23] to 16.

Materials and Methods

Animal study

Animals and Administrative Procedure

Thirty 4-week-old female Wistar rats were supplied by CLEA Japan, and housed in a room under a 12-h light / 12-h dark cycle at 23°C. The rats were maintained with free access to γ-ray-sterilized CE-2 laboratory powdered chow (CLEA Japan) containing MeHg (5 *u*g/g of Hg). This level of MeHg caused no decrease in body weight or apparent toxicity symptoms in our previous experiment.[15] In the previous experiment, the daily uptake of the diet was restricted to 16 g per rat until mating, but this time we maintained the rats with free access to the diet. The MeHg exposure level was monitored by measuring Hg concentration in blood (described in the next subsection) and when the level had almost reached a plateau, the females were mated with males. The pregnant females were then continued on the same diet, with access *ad libitum*, throughout the gestation and suckling periods until postnatal day 20 (P20). The MeHg exposure level in the mothers and offspring were also monitored throughout late gestation and after parturition by measuring Hg concentrations in the blood and brain.

Samples

About 5-10 μL of blood was withdrawn from the tail vein of 4 randomly chosen females in 30, fed on the MeHg-containing diet, every two weeks until mating to monitor the MeHg exposure level at various stages before mating, during gestation and late gestation. On embryonic days 18, 20, 22 (E18, E20, E22) 3 mothers and 6 fetuses (two fetuses from each litter) were randomly chosen to be sacrificed to determine the tissue concentration of Hg. Another, randomly chosen group of 3 mothers and 6 infants (2 females and 2 males each from each litter) were sacrificed on the day of parturition and postnatal days 10 and 20 (P10, P20). On postnatal days 5 and 15 (P5, P15) 8 infants (4 male and 4 female offspring) were also sacrificed to determine tissue Hg concentrations. For tissue sampling, rats were deeply anesthetized by an intraperitoneal injection of pentobarbital. Blood samples were collected by cardiac puncture, and the rats were then killed by transcardiac perfusion with physiological saline for 5 min to flush out blood from the brain. The removed tissues were kept at -80°C until use.

Mercury Determination Procedure

Total Hg concentrations in the tissues were determined according to the oxygen combustion-gold amalgamation method[29] using a Mercury Analyzer MV 250R (Sugiyama-gen Environmental Science Co., Tokyo). The total analytical precision for this analysis was estimated to be 3.9% and 1.0% at mercury concentrations of 2.6 μg/g and 11.1 μg/g, respectively. Accuracy was ensured by using certified reference material (DORM-2; Dog fish muscle prepared by the National Research Council, Canada) as quality control material; the Hg concentration averaged 4.78 μg/g, as compared to the assigned value of 4.64

± 0.26 μg/g.

Human Study

Subjects

In the previous study[23], the number of the subjects was 7. We added another 9 subjects. In total sixteen healthy Japanese pregnant women, ranging in age from 22 to 36 yr (average 30.4 ± 4.3 yr), planning to deliver in Munakata Suikokai General Hospital, Munakata City, Fukuoka, Japan, gave informed consent to take part in the present trial. In all infants, five of the infants are male. The average body weights at birth and the age of after 3 months were 3.3 ± 0.32 and 6.36 ± 0.62 kg, respectively. During the study, five mothers had delivered their first child, three their second, and eight their third. Two mothers consumed fish once everyday, the others of them answered that two or three times per week. Fifteen of the infants were reared on breast milk. Only one was reared mainly on breast milk and additional milk formula beginning at 4 and 6 weeks of age.

Ethics and informed consent

Human study was approved by the Ethics Committee of NIMD (National Institute for Minamata Disease). Sixteen normal Japanese pregnant women without any special exposure to mercury, and living in Munakata City, Fukuoka, Japan, gave their informed consent to take part in the trial.

Samples

Blood samples were collected from sixteen pairs of mothers and infants. The samples included 13 ml of venous umbilical cord blood at birth and 10 ml of venous maternal blood 1 day after parturition before breakfast, 2 ml of each infant blood at 3 months of age. All blood samples were obtained by venipuncture with a small amount of heparin-Na and centrifuged at 3000 rpm for 10 min to separate into RBCs. Samples were stored at –80 ^{o}C until analysis.

Hg Analysis

Total Hg in 0.5 g of RBCs was determined by cold vapor atomic absorption spectrophotometry (CVAAS) according to the method of Akagi and Nishimura.[30] The method involves sample digestion with HNO_3, $HClO_4$, and H_2SO_4 followed by reduction to Hg^0 by $SnCl_2$. Accuracy was ensured by using certified reference material (DORM-2; Dog fish muscle prepared by the National Research Council Canada) as quality control material; the Hg concentration found averaged 4.53 μg / g, as compared to the assigned value of 4.64 ± 0.26 μg / g. Total analytical precision of this analysis was estimated to be 3.9%.

Statistical analysis

Hg concentrations were represented by means ± SD. Student's *t*-test and Paired *t*-test were used for the statistical analysis of the data. A *P* value less than or equal to 0.01 was considered

to demonstrate statistical significance.

Result

Animal study

Hg concentrations in blood

The time-course changes in Hg concentrations measured in whole blood of the females before pregnancy and after parturition, as well as of the delivered offspring are depicted in Figure 1. The Hg concentration of the females increased with the duration of administration, and reached a near plateau after 8 weeks. The Hg concentration of the mothers decreased throughout gestation, and at parturition fell to approximately 50% of that in the mating period. After 20 days of lactation, the Hg concentration of mothers resembled that in the mating period. On the other hand, the time-course changes in Hg concentration of the offspring showed a pattern different from that of the mothers. On the day of birth, the concentration in blood was significantly ($p<0.01$ by Student's *t*-test) higher, i.e., approximately 2 times that of their mothers on that same day. However, that concentration rapidly decreased throughout their suckling period. All offspring grew up without any physical signs of typical MeHg poisoning, such as ataxia or hind-limb crossing.

Hg concentrations in the brain

We also measured the changes in Hg concentration in the brain tissue of both mothers and offspring (Fig. 2). The patterns of time-course changes in the brain were similar to those in the blood. The average concentrations in the brain on fetal days E18, E20, E22 and at birth were about 4-4.5 μg/g, which was about 1.5 to 2 times higher than those in the brain of the mothers ($p<0.01$ by Student's *t*-test). That of offspring concentration rapidly decreased during the suckling period down to about 1 / 10 of that at parturition.

Human study

Hg concentrations in red blood cells in infants at birth and 3 months of age

At birth, RBCs-Hg levels in umbilical cords were higher than those in mothers in all sixteen cases. The mean of RBCs-Hg of umbilical cord was 13.0 ng/g, which was significantly higher than in mothers (8.19 ng/g) by paired *t* test ($p<0.01$; Fig. 3). A strong correlation was observed in RBCs-Hg in mothers and umbilical cords at parturition ($r = 0.96$, $p <0.01$, Fig. 4).

Although most of the mothers said that the amount and the species of fish consumed did not change during the lactation period, in all sixteen infants the Hg concentrations decreased throughout this period. The mean of RBCs-Hg in infants at 3 months of age was 6.87 ng/g, significantly lower than that at birth (13.0 ng/g) by paired t test ($p<0.01$; Fig. 5).

Discussion

Animal study

This study was designed so that fetuses conceived in females that had been exposed to a constant and consecutive dose of MeHg before and throughout gestation were exposed to MeHg transplacentally throughout the entire gestational period, followed by post-partum exposure through contaminated milk. This was considered to simulate the natural course of offspring exposure to MeHg among people who commonly consume a lot of fish and sea mammals, and to reveal some new aspects of the risk of MeHg to offspring.

The concentrations of Hg in the brain of fetus were 1.5 to 2 times higher than those in their mothers throughout late gestation (Fig. 2). This is in accordance with the proposal that MeHg is actively transferred to the fetus across the placenta via neutral amino acids carriers[10,13] throughout the late gestation period. The decrease in Hg concentrations in maternal blood during the gestation period partly explains the accelerated MeHg transfer to the fetus according to the demand of amino acids to promote fetal growth. It is known that the developing brain is most vulnerable the toxic effect of MeHg during the third-trimester.[6,17] Our results indicate that the Hg concentrations in offspring brain were higher than that in maternal brain not only at parturition but also throughout last gestation period when the human brain is most vulnerable.

We also demonstrated that Hg accumulations in offspring differ significantly between periods of gestation and suckling. During the 20 days after birth, the offspring concentrations in the blood and brain decreased dramatically as demonstrated in a previous animal experiment.[15,31-33] This will be explained by limited MeHg transfer from milk[32,34,35] and rapid increase in the organ and body volume. Further, the increase in blood Hg concentrations in maternal blood during suckling can be partly explained by the diminished MeHg transfer to the fetus compared with that during the gestation period.

Human study

We increased the number of the subjects from 7 in the previous study[23] to 16. The changes in RBCs-Hg concentration at birth and 3 months of age were investigated. This study was designed mainly to determine the changes in MeHg levels in infants after parturition, followed by further MeHg exposure through milk after birth. RBCs-Hg can be used as a biomarker of MeHg exposure.[3,38,39] It is known that the RBCs to plasma ratio of Hg concentration is approximately 1:1 in nonfish-consuming populations and after exposure to Hg^0 vapor.[3,39] However, in general the higher the fish consumption (MeHg exposure) the higher the RBCs to plasma ratio, which reaches approximately 8-9:1 in populations that consume much fish.[,3,23,39-41] Additionally, more than 80% of Hg in the total blood[42,43] and more than 90% of that in RBCs is known to be in the methyl form[43] in high-fish-consumptions, indicating that Hg source was predominantly MeHg from fish. RBCs-Hg is one of the best biomarker to

determine the MeHg exposure level.[23,37,43-45] Therefore, the changes in MeHg levels infants during breast-feeding were investigated using the total Hg concentrations in RBCs in this and previous[23] studies.

For the reasons mentioned below, the number of amalgam filling was not investigated. Our objective was to determine the changes in MeHg levels in infants after parturition using RBCs-Hg, which was hardly influenced by amalgam filling in this high-fish-consumption population. In Japan, the number of amalgam filling is low and has been calculated to be about 2 in 100 people at present.[46] Since the amount of fish consumption is much higher than that in typical Europeans and Americans, the effect of amalgam fillings will be slight even in Plasma-Hg level in Japanese.

RBCs-Hg levels in umbilical cords were about 1.6 times higher than those in the mothers, and there was a strong correlation between them at birth. This suggests that MeHg actively transfers to fetus across the placenta via neutral amino acids carrier, as demonstrated by Kajiwara.[13] This higher Hg accumulation in the fetuses than in mothers is widely acknowledged we mentioned in the animal study. In addition, the susceptibility of the developing brain itself is high.[3,15,43,44] Thus, the risk of exposure of the fetus to MeHg is very high.

After 3 months of breast-feeding the RBCs-Hg in infants dramatically declined to 53% of that at birth (Fig. 5). The contribution of breast milk to MeHg transfer to infants seems limited, as was recently suggested by Sandborgh-Englund *et al*.[47] and Sakamoto et al.[23] Once neonates are separated from the active intrauterine amino acid transport system. Hg transfer depends on the milk, in which the Hg concentration is about 20% that in maternal plasma.[23] During this period, the average body weight of infants quickly increases and becomes about 1.9 times that at birth. Consequently, the average body volume and the limited Hg transfer from breast milk might have caused the dilution in RBCs-Hg levels during this period. However, the contribution of the excretion of MeHg may be small, judging from the diminished MeHg excretion in infant animals.[3]

In conclusion, though maternal MeHg can be transferred to infants through breast milk, following exposure through placenta during their intrauterine life. The risk to the offspring might be especially high throughout the late gestation period but rapidly decreases during the suckling as indicated by both the animal and human studies. Thus, sufficient attention should be paid on the gestation rather than on the breast-feeding period to avoid dangers of MeHg to human infants. Therefore, if exposure levels are constant and low enough not to cause adverse effects on fetuses during gestation, mothers need not worry about the breast-feeding. The benefits of breast-feeding [23,36,37] may well be greater than the possible adverse effects of MeHg in breast milk under such conditions. However, if mothers were exposed to high MeHg levels during suckling, caution is recommended concerning breast-feeding.[23]

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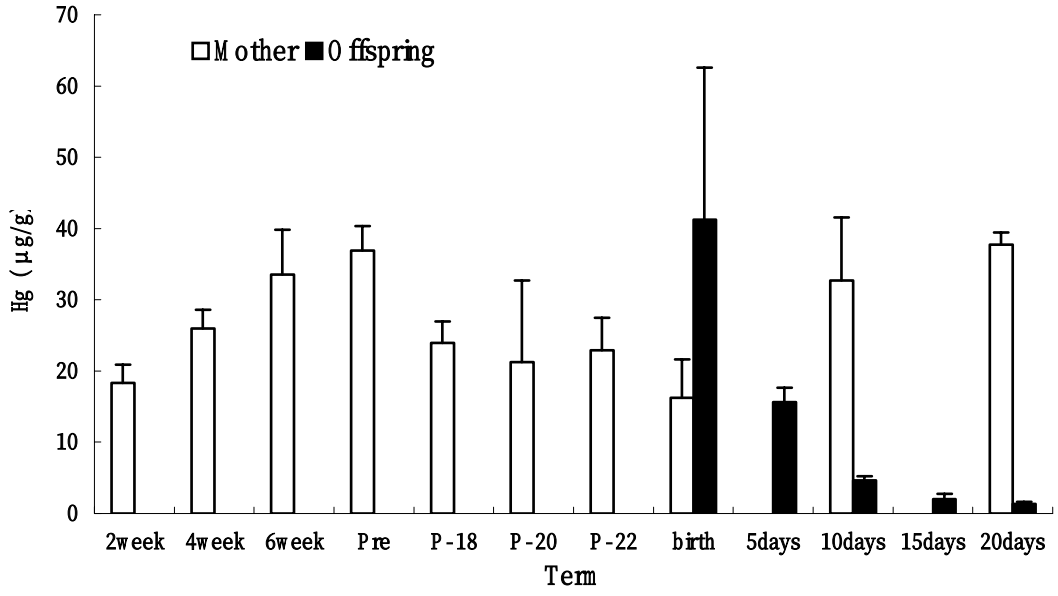

Figure 1 Time-course changes in Hg concentrations in the whole blood of female rats during 8 weeks before and during pregnancy as well as after parturition and those in their offspring at birth and during suckling. For 8 weeks, female rats were fed a MeHg-containing diet (5 μg/g Hg as MeHg). They are then mated and continuously fed this diet during the gestation and lactation periods. The offspring are fed on mother milk until weaning on postnatal day 20. Data represent means±*SD* for mothers (n=3-4) and infants (n=4-6).

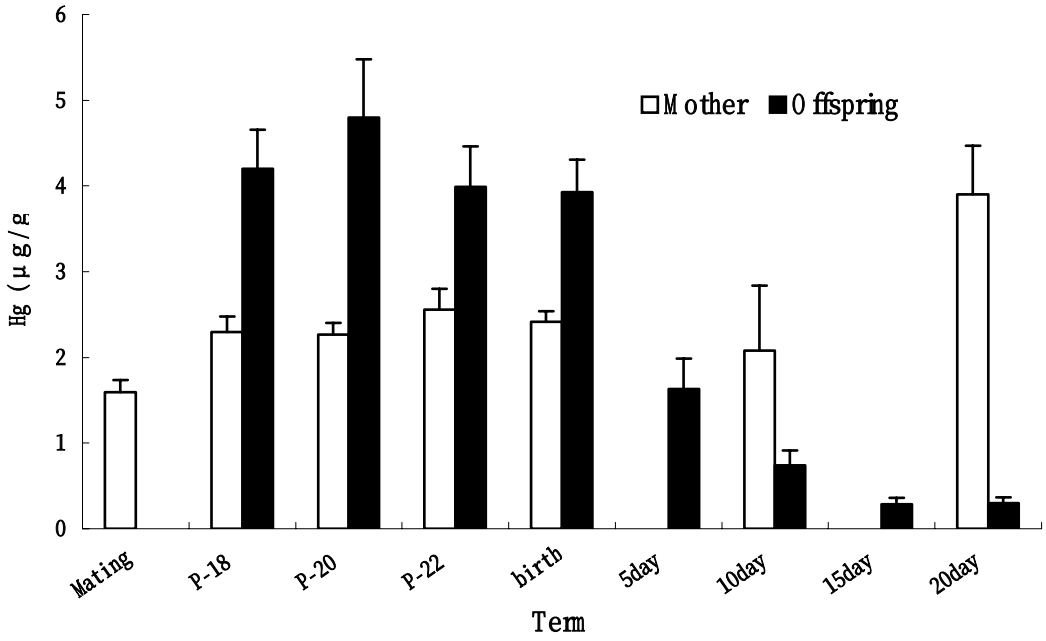

Figure 2 Time-course changes in Hg concentration in the brain of maternal rats during late gestation and suckling and those in offspring at during late gestation and during suckling. Data represent means±*SD* for mothers (n=3) and infants (n=6).

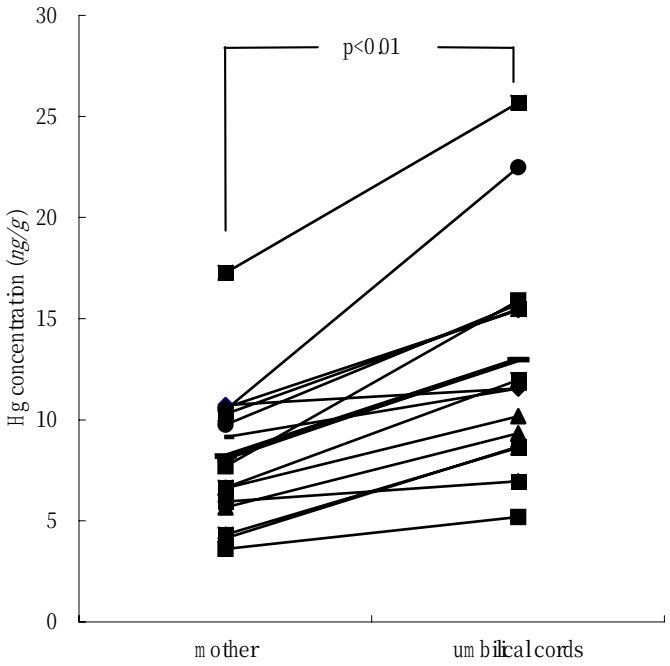

Figure 3 Comparison of mercury concentrations in maternal and umbilical cord red blood cells. Fetal RBCs-Hg was significantly higher than those of maternal at birth by paired *t* test ($p<0.01$). The horizontal lines indicate the means.

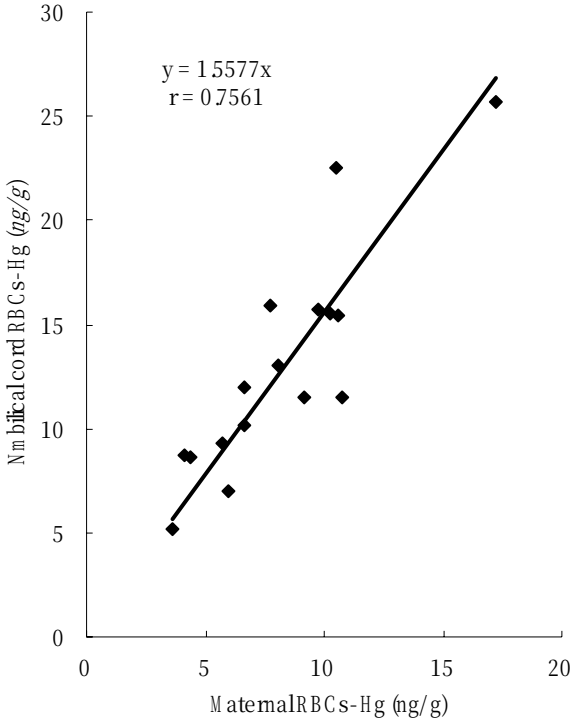

Figure 4 Correlation between maternal and umbilical cord Hg concentrations in red blood cells in 16 maternal-fetal pairs. In all 16 cases umbilical cord RBCs-Hg levels were higher than maternal levels. A strong correlation was observed in RBCs-Hg between mothers and umbilical cord (r=0.76, $p < 0.01$).

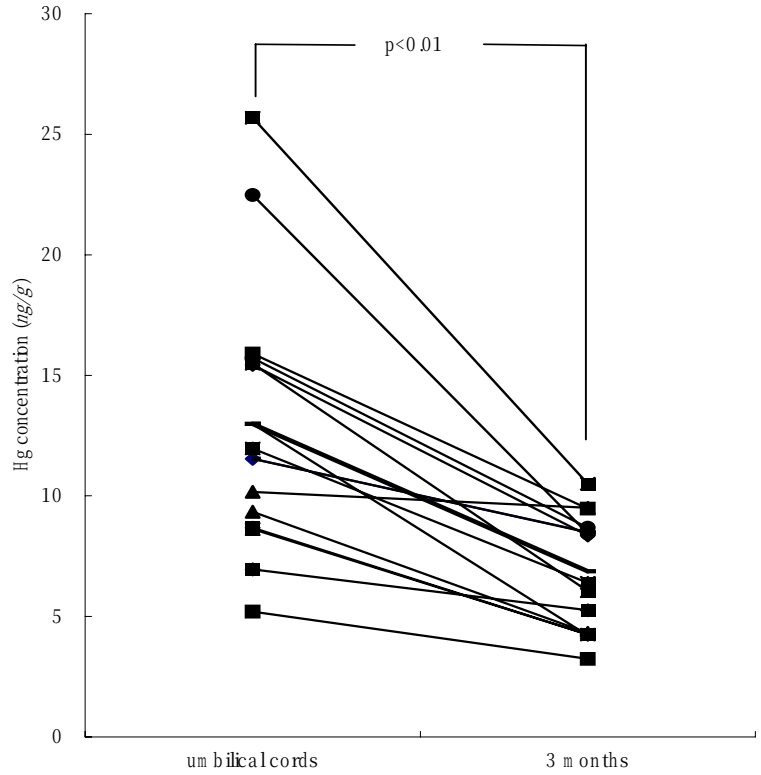

Figure 5 Changes in mercury concentration in RBCs from umbilical cord to those in infants at 3 months of age. The infant RBCs-Hg at 3 months of age was significantly lower than those at birth by paired t test ($p < 0.01$). The horizontal lines indicate the means.

[F-6]

RECENT TRENDS IN METHYLMERCURY AND CADMIUM RISK ANALYSIS

Rie Masho

Center for Environmental Information Science

1. Grobal Food Standards--*Codex Alimentarius*

The global reference in food quality and safety, *Codex Alimentarius* is created by the *Codex Alimentarius* Commission (CAC)(Table 1). The CAC was established in 1963 by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) to consult the director generals of these organizations on the implementation of the Food Standard Program. As of 2004, 170 countries including Japan participate in the CAC .

Among the Codex Committees, subsidiary bodies that prepare proposals for the CAC, the Codex Committee on Food Additives and Contaminants (CCFAC) is responsible for establishing maximum or guideline levels for individual food additives, contaminants, and naturally occurring toxicants in food and animal feed (1).

2. Risk Analysis under *Codex Alimentarius*

In the *Codex Alimentarius* framework, food related risks are handled according to the principles for “Risk Analysis”, which consists of three closely related but functionally separated components, “Risk Management”, “Risk Assessment”, and “Risk Communication” (Fig. 1)(2). The first step of risk analysis is risk evaluation: risk managers decide if particular issue is significant and needs further assessment. The second step is risk assessment: experts quantify the risks associated with specific contaminants or management options on the basis of science. The third step is the policy decision: consulting risk assessment results, risk managers choose an option, implement it, monitor its effects and reevaluate the options. In all risk analysis processes, risk communication amongst risk assessors and risk managers, and with member countries and all interested parties needs to be included.

The CCFAC decides upon which food additives/contaminants require toxicity and/or exposure assessments by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), an international scientific committee administered jointly by the FAO and the WHO. The members of the JECFA are appointed by the two organizations from the rosters of pre-registered scientists, asked to participate solely on their individual capacities as experts, and not allowed to represent interests of his country, institute or company. CCFAC proposes maximum levels of food additives and contaminants based on scientific consultation of JECFA and the proposals are made *Codex Alimentarius* standards after adopted by CAC (Fig.

2).

3. Scientific Evaluations and Standards for Methylmercury and Cadmium

Recent scientific evaluations of cadmium and methylmercury took place in the 61st JECFA meeting in June 2003 (3). The provisional tolerable weekly intake (PTWI) for cadmium was assessed at 7 μg/kg bw, maintaining the previous value. The PTWI for methylmercury was assessed at 1.6 μg/kg bw, half the previous value. Considering the new PTWI for methylmercury in its 36th session in March 2004, the CCFAC decided to resume a discussion on methylmercury standards (4). A working group is established under the direction of the European Community to prepare a discussion paper on the possible revision of the guideline levels for Methylmercury in fish for consideration at the 37th CCFAC session in April 2005 (Table 2). Proposals for the maximum levels of cadmium for polished rice, wheat grain, potato, certain vegetables, and molluscs are also kept on the agenda for the 37th CCFAC session. On the basis of the JECFA's maintained cadmium PTWI, the 36th CCFAC proposed to the CAC the maximum level of 0.4 mg/kg for cadmium in polished rice, the same level as Japanese national guideline bans circulation (4). Since Japanese rice sometimes contains elevated levels of cadmium, the decision by the CAC was of great interest in Japan. The CAC, however, sent the proposal back to the CCFAC for further consideration, due to the concern that the maximum level proposed could result in intakes exceeding the PTWI in certain populations (5). The CAC requested the CCFAC to take careful account of the results of the JECFA's cadmium exposure assessment which is going to take place in February 2005 and also to encourage member countries to provide data to JECFA to facilitate its assessment (Table 3).

4. *Codex Alimentarius* and National Legislations

Guideline/maximum levels by *Codex Alimentarius* are not legally binding by themselves. However, they are the single reference in food safety when The World Trade Organization (WTO) deals with the rules of trade between nations. This gives a good reason for governments to set their national regulations accordingly. Besides the purpose of protection against trade conflicts, governments establish national regulations/guidelines to protect their people. While Japanese maximum level for cadmium in unpolished rice is 1.0 mg/kg, the government removes from circulation the crop containing 0.4 mg/kg of cadmium or higher (6). Japanese government has also established, in similar efforts as many of other countries, guidelines on fish consumption for young women and children to protect them from excessive methylmercury intake (7).

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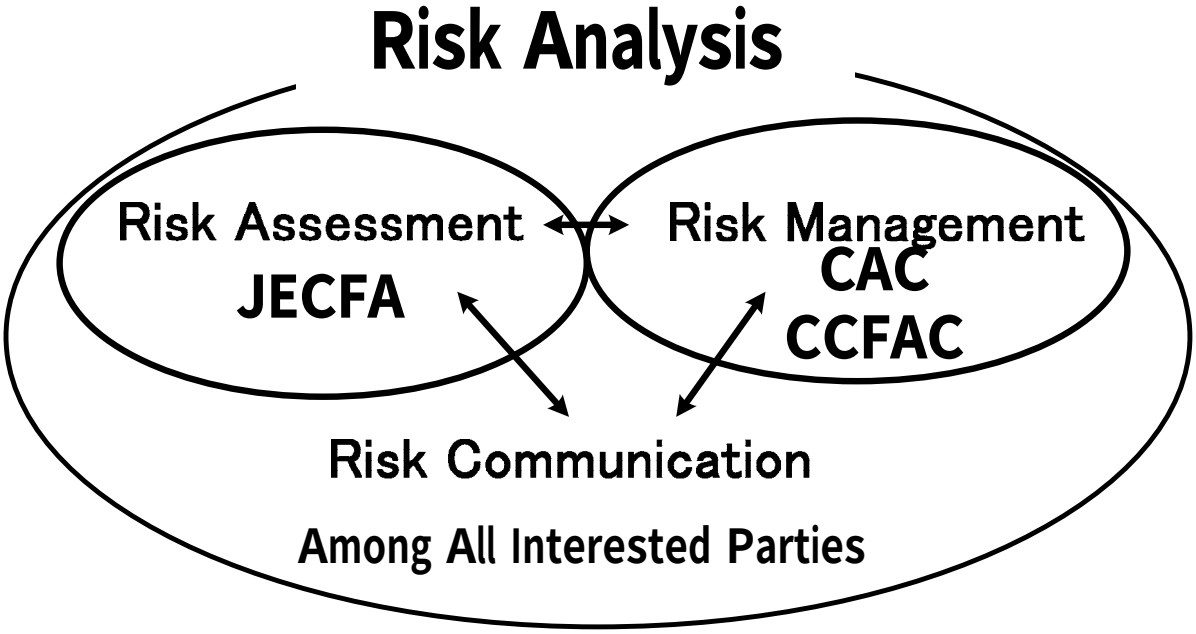

Fig.1 Framework of Risk Analysis under *Codex Alimentarius*

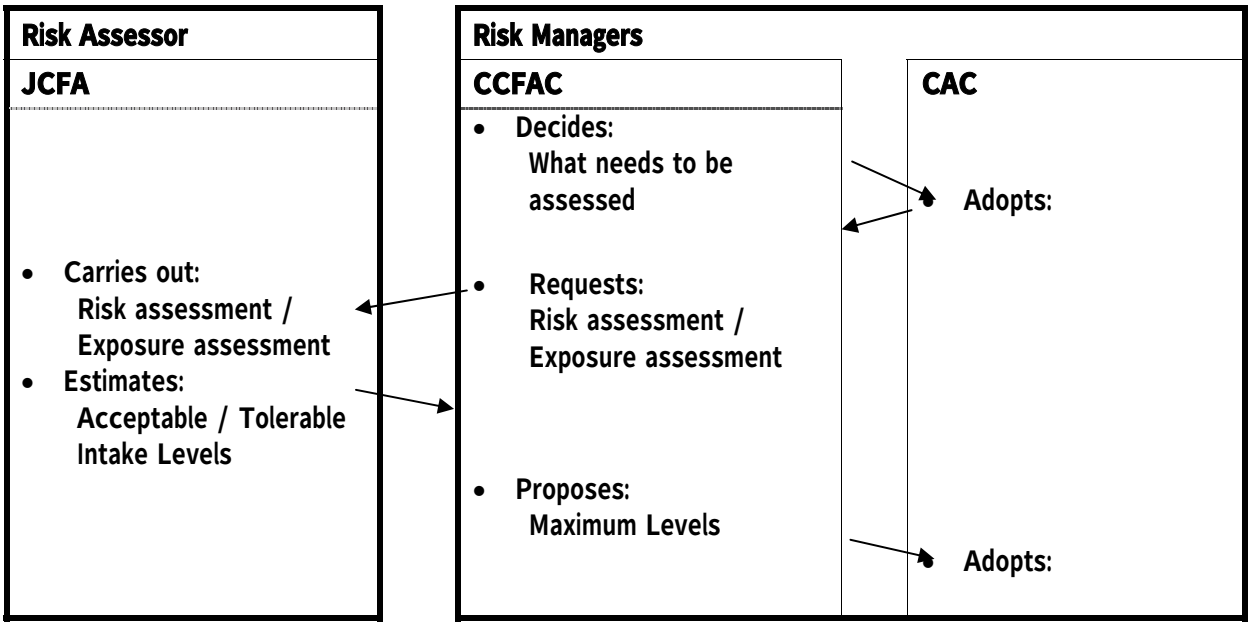

Fig. 2 Three Entities Responsible for Standard Setting in *Codex Alimentarius*

Table. 1 Working Principals under *Codex Alimentarius*

Maximum Levels (MLs) Shall Be Set:
• on Contaminants that Present: Significant Health Risk Problems in International Trade • on Foods that cause: Significant Exposure • as Low as Reasonably Achievable (ALARA Principle) • Based on data (Risk / Exposure Assessment)

Table 2 Methylmercury Standards and Concerns

***Codex Alimentarius* Standards for Methylmercury:**

- Guideline Levels in fish (1991)

All Fish Except Predatory Fish	0.5 mg/kg
Predatory Fish	1 mg/kg

Concerns:

- Which are the Predatory Species?
- More Strict MLs Needed?
- New TWI by JECFA (2003)
  PTWI 0.0016 mg/kg bw
  (1988-2002: PTWI 0.0033 mg/kg bw)

Table 3 Cadmium Standards and Concerns

***Codex Alimentarius* Standards for Cadmium in Rice:**

- Draft Maximum Levels (At Step 3 of 8)

Rice, Polished	0.4 mg/kg

Concerns:

- Draft ML for Polished Rice was 0.2 mg/kg (Step3. CCFAC, 2003)
- It was changed to 0.4 mg/kg according to Japanese Proposal. (Step 5. CCFAC, 2004)
- The proposal was sent back to Step 3 by CAC, 2004.
- The Draft ML Could Result in Intake Exceeding the PTWI→

  Should Take account of the JECFA ' s Evaluation (Feb. 2005)

[F-7]

CHRONIC EXPOSURE TO CADMIUM AND HEALTH EFFECTS IN INHABITANTS OF KAKEHASHI RIVER BASIN, ISHIKAWA PREFECTURE, JAPAN

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The Kakehashi River basin, Ishikawa Prefecture, Japan was polluted by cadmium (Cd) compounds, which were transported by this river from a mine upstream to the rice fields where river water was used for irrigation. Two major epidemiological studies were conducted in 1974-75 and 1981-83. Urinary β_2-microglobulin (β_2-MG) was primary used for an indicator of renal tubular dysfunction to all the participants consisted of 3,178 inhabitants all over 50 years of age living in the Cd-polluted areas and 294 in 2 Cd-unpolluted areas. Prevalence of β_2-MG-uria (> 1,000 μg / g cr.) was 14.3 % in men and 18.7% in women in Cd-polluted areas, while it was 6.0 % and 5.0 % in men and women in unpolluted areas, respectively (Table 1). [1,2] The previous days, concurrent prevalence of proteinuria and glucosuria were used at the first stage of screening test for Cd-induced renal damage. If the same procedure was conducted in this epidemiological study, only 12.4 % of β_2-MG-uria above was found as renal tubular dysfunction.

As urinary indicators other than β_2-MG, α_1-MG, metallothionein (MT), N-acetyl-β-D-glucosaminidase (NAG) and intestinal alkaline phosphatase (IAP) were utilized (Table 2). [3-6] Urinary β_2-MG, α_1-MG and MT reflect renal tubular function, while NAG and IAP in urine indicate damage of renal tubular cells. They are relatively related each other, although NAG and IAP gradually decrease at severe stage of renal tubular damage induced by Cd. β_2-MG shows wide range of Cd-induced renal tubular dysfunction. Therefore, urinary β_2-MG is one of the most useful indicators for the detection of renal tubular dysfunction.

Once Cd-induced renal tubular dysfunction occurs, it was irreversible even after cessation of Cd-exposure (Table 3). [7] Follow-up studies over 20 years showed elevation of β_2-MG, even though urinary Cd slightly decreased. About half of them showed increase in serum creatinine and decrease in arterial blood pH, which resulted in glomerular dysfunction and metabolic acidosis (Table 4). [8]

Cd-induced bone damage was also examined using microdensitometry (MD). Several indicators of this MD methods shows osteopenia and it was significantly low density in women exposed to Cd compared with women in unpolluted areas These indicators significantly associated with urinary β_2-MG or serum creatinine by multiple regression

analysis (Table 5). [9, 10] At least, three residents with severe renal tubular dysfunction were diagnosed as osteomalasia by autopsy or bone biopsy.

The relationship between prevalence of β_2-MG-uria and urinary Cd was shown using probit analysis. As results, urinary Cd concentration corresponding to prevalence of β_2-MG-uria in inhabitants living in the unpolluted areas was 3.8 – 4.0 μg / g cr. in men and 3.8 – 4.1 μg / g cr. in women, respectively. Using urinary MT instead of urinary β_2-MG, allowable Cd concentration was calculated as 4.2μg / g cr. in men and 4.8 μg / g cr. in women (Table 6). [11, 12]

Urinary Cd concentration was significantly correlated with total Cd intake calculated by formula as follows; ([village average Cd concentration in rice] × [the average daily intake of rice] + [daily intake of Cd from foods other than rice]) × [duration of residence in the Cd-polluted areas] + [average daily intake of Cd in non-polluted areas of Japan] ×[duration of residence in non-polluted areas]. The correlation coefficients between urinary Cd concentration and total Cd intake was 0.93 in men and 0.88 in women on a group basis. [13] Based on individual data of Cd in urine, the correlation coefficients was 0.88 and 0.84 in men and women, respectively. [14]

Total Cd intake corresponding to maximum allowable Cd concentration in urine was calculated as approximately 2 g for both of men and women using linear regression analysis. To consider the effect of age, multivariate analysis such as logistic regression analysis and general linear models were used and a variable of total Cd intake was independently associated with prevalence of β_2-MG-uria or MT-uria. These total Cd intake values were calculated as 1.5 -2.6 g and close to those calculated by simple regression analysis (Table 7). [15-18]

Consequently these results suggest that 2 g is reasonable value as the maximum allowable intake of Cd.

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Table 1 Prevalence (%) of abnormal urinary findings in Cd-exposed and nonexposed subjects[9,10].

Age	Cd-exposed subjects					Nonexposed subjects				
	50-59	60-69	70-79	80-	Total	50-59	60-69	70-79	80-	Total
Men										
N	600	494	265	65	1,424	62	38	26	7	133
Glucose≧20mg/dl	1.3	2.6	4.2	7.7	2.6	4.8	0	0	0	2.3
with protein≧5mg/dl										
Amino acids≧300mg/g.cr	0.7	1.6	3.0	9.2	1.8	1.6	2.6	0.0	0.0	1.5
β_2-MG≧1000μg/g.cr	4.8	13.0 **	28.7	52.3	14.3 **	0	0	26.9	14.3	6.0
MT≧638μg/g.cr	1.5	6.5	7.5	6.2	4.6	4.9	0	0	0	2.3
Women										
N	713	591	340	110	1,754	64	49	34	14	161
Glucose≧20mg/dl	0.6	1.9	7.1	20.0	3.5	0	4.1	0	7.1	1.9
with protein≧5mg/dl										
Amino acids≧300mg/g.cr	5.9	7.8	10.6	23.6 *	8.6 **	1.6	2.0	2.9	0	1.9
β_2-MG≧1000μg/g.cr	4.9 *	17.1 *	36.5 **	61.8 **	18.7 **	0	6.1	5.9	21.4	5.0
MT≧693μg/g.cr	4.5	10.2	10.9	16.5	8.4 *	0	10.2	0	0	3.1

N: Number of persons examined.

*: Significant difference from control ($p<0.05$).

**: Significant difference from control ($p<0.01$).

Table 2 Urinary findings in Cd-exposed and nonexposed subjects.

	Cd-exposed subjects				Nonexposed subjects		
	Sex	N	Mean	S.D.	N	Mean	S.D.
β_2-MG	M	67	7,116	6.38 **	26	141	3.87
(μg/g.cr.)	W	102	10,934	5.11 **	55	174	3.47
α_1-MG	B	27	18,880	5.5 **	10	352	4.2
(μg/g.cr.)							
NAG	M	39	51.1	2.45 **	22	25.3	1.60
(U/g.cr.)	W	36	43.9	2.21 *	26	27.2	1.88
hIAP	M	18	4.6	2.07 **	18	1.26	1.75
(IU/g.cr.)	W	22	4.7	2.99 **	22	1.82	2.28
Mucoprotein	M	67	228.6	1.82 **	26	75.9	1.75
(mg/g.cr.)	W	102	309.2	1.84 **	55	81.5	1.90
Albumin	M	67	93.6	4.79 **	26	29.3	2.47
(mg/g.cr.)	W	102	140.0	3.60 **	55	31.7	2.80
Total Protein	M	67	185.6	3.62 **	26	68.3	1.81
(mg/g.cr.)	W	102	251.7	2.73 **	55	73.4	2.01
Cd	M	67	7.5	1.82 **	26	2.5	1.58
(μg/g.cr.)	W	102	10.1	1.74 **	55	4.0	1.45

N: Number of persons examined.

M: Men, W: Women, B: Both Sexes.

Mean, S.D.: Geometric mean and geometric standard deviation.

*: Significant difference from controls ($p<0.05$).

**: Significant difference from controls ($p<0.01$).

Table 3 Urinary finding of environmentally Cd-exposed inhabitants examined in 1981 and1986[32].

	1981			1986		
	N	Mean	S.D.	N	Mean	S.D.
Glucosuria (mg/g.cr)	74	164.4	2.79 **	74	262.7	2.58
Aminoaciduria (mg/g.cr)	74	188.3	1.60 **	74	213.2	1.77
β_2-microglobulinuria (μg/g.cr)	74	857.6	9.06 *	74	1,252.1	12.91
Cd (μg/g.cr)	67	10.0	1.87	74	9.6	1.74

Mean S.D.: Geometric mean & geometric standard deviation.

*: Significant difference between the data obtained in 1981 and 1986 ($p<0.05$).

**: Significant difference between the data obtained in 1981 and 1986 ($p<0.01$).

Table 4 Time-related trends in mean serum creatinine and in mean arterial blood pH of the Cd-exposed subjects[34].

	N	At initial examinaton		At time Cd was removed		At most recent examination	
Creatinine in serum[a] (mg/g cr)	21	1.19	1.28**	1.22	1.37*	1.68	1.56
Arterial blood pH[b]	21	7.400	± 0.023**	7.386	± 0.034**	7.361	± 0.058

[a]: Geometric mean & geometric standard deviation.

[b]: Arithmetric mean & arithmetric standard deviation.

*: Significant difference ($p<0.05$) compared with the Value at the most recent examination.

**: Significant difference ($p<0.01$) compared with the Value at the most recent examination.

Table 5 Biological parameters selected by stepwise backward regression analysis and significance of their standard partial regression coefficients to microdensitometrical indices[42].

	Cd-exposed men (N=91, Mean Age ; 70.8)						Cd-exposed women (N=112, Mean Age ; 70.7)					
	MCI	d	GSmax	GSmin	ΣGS/D	Sum of given scores	MCI	d	GSmax	GSmin	ΣGS/D	Sum of given scores
Age	*			**	*		**	**		**	**	
log (U-Cd/Cr)												
log (U-β_2-mg/Cr)	**	**	*	**	**	**	*	*	**	**	**	**
log (S-Cr)	**	**	**	**	**	**						
S-Ca		*				*						
S-P												
log (B-Cd)												
R	0.51	0.51	0.43	0.50	0.43	0.47	0.53	0.42	0.35	0.49	0.44	0.25
	**	**	**	**	**	**	**	**	**	**	**	**

	Nonexposed men (N=25, Mean Age ; 72.4)						Nonexposed women (N=55, Mean Age ; 69.0)					
	MCI	d	GSmax	GSmin	ΣGS/D	Sum of given scores	MCI	d	GSmax	GSmin	ΣGS/D	Sum of given scores
Age								**			**	*
log (U-Cd/Cr)			*									
log (U-β_2-mg/Cr)												
log (S-Cr)									*			
S-Ca		*										
S-P												
log (B-Cd)								*	*			
R	0.45	0.60	0.43	0.48		0.41	0.41	0.37	0.37	0.54	0.46	0.38
							**	*		**	**	*

R: Multiple correlation coefficient.

Table 6 Probit linear regression lines for urinary Cd concentrations and prevalences of β_2-microglobulinuria or metallothioneinuria, and urinary Cd concentrations corresponding to prevalence rates of β_2-microglobulinuria or metallothioneinuria in the control population.

Sex	Indicators	Cutoff levels	Probit regression lines	Urinary Cd concentrations[a)] (μg/g.cr)	Prevalence rates of each control[b)] (%)
Men	β_2-MG	927μg/l	Y=2.58X+1.84	4.0	5.3
	β_2-MG	1,129μg/g.cr	Y=2.40X+2.05	3.8	6.0
	MT	645μg/g.cr	Y=2.35X+1.44	4.2	1.8
Women	β_2-MG	503μg/l	Y=2.33X+1.34	3.8	4.3
	β_2-MG	1,059μg/g.cr	Y=2.38X+1.94	4.1	5.0
	MT	738μg/g.cr	Y=1.66X+2.00	4.8	3.1

Y: Prevalence of β_2-microglobulinuria or metallothioneinuria (Probit value).

X: Log (μg Cd/g creatinine in urine).

a): Urinary cadmium concentration corresponding to each control's prevalence rate of β_2-microglobulinuria or metallothioneinuria.

b): Prevalence rate of β_2-microglobulinuria and metallothioneinuria in control subjects.

Table 7 Equations relating total Cd intakes and prevalences of β_2-microglobulinuria and metallothioneinuria, and total Cd intakes correnponding to prevalence rates of β_2-microglobulinuria and metallothioneinuria in the control population.

Sex	Indicators	Cutoff levels	Equation	Total Cd intake (g)	Prevalence rates of each control[b)] (%)	Statistical analysis
Men	β_2-MG	1,000μg/l	Y=7.6X-10.3	2.06	5.3	Linear regression
	β_2-MG	1,000μg/g.cr	Y=8.3X-7.9	1.68	6.0	Linear regression
	MT	645μg/g.cr	Y=3.3X-4.8	1.99	1.8	Linear regression
	MT	535μg/l	Y=0.58X+0.011(Age)-5.7	2.22	2.5	Logistic regression
	MT	628μg/g.cr	Y=0.58X+0.020(Age)-6.3	2.21	2.5	Logistic regression
	MT	535μg/l	Y=4.4X-0.080(Age)-3.1	2.41	2.5	General linear models
	MT	628μg/g.cr	Y=5.9X-0.071(Age)-11.4	2.65	2.5	General linear models
Women	β_2-MG	1,000μg/l	Y=11X-19.6	2.07	3.1	Linear regression
	β_2-MG	1,000μg/g.cr	Y=12X-16.2	1.76	5.0	Linear regression
	MT	738μg/g.cr	Y=6.3X-11.1	2.26	3.1	Linear regression
	MT	535μg/l	Y=0.57X-0.015(Age)-4.0	2.37	2.5	Logistic regression
	MT	628μg/g.cr	Y=0.57X+0.012(Age)-4.7	0.32	2.5	Logistic regression
	MT	535μg/l	Y=4.4X-0.080(Age)-2.5	2.56	2.5	General linear models
	MT	628μg/g.cr	Y=5.9X-0.071(Age)-18.2	1.51	2.5	General linear models

Y: Prevalence of β_2-microglobulinuria and metallothioneinuria.

X: Total Cd intake (g).

[F-8]

RISK EVALUATION OF CADMIUM BASED ON THE EPIDEMIOLOGICAL STUDIES IN CADMIUM-POLLUTED OR CADMIUM- NONPOLLUTED AREAS

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Cadmium (Cd) is the causative agent of itai-itai disease, outbreaks of which have been reported in at least four of the Cd-polluted regions of Japan [1-3]. An early finding of health effects caused by exposure to Cd is renal tubular dysfunction, characteristically manifested by increased excretion of low molecular weight proteins, notably β2-microglobulin (β2-mg). The major significance of this finding is the observation based on a 9-year follow-up of inhabitants of Cd-polluted regions that life prognosis is adversely affected when normal excretion levels of these proteins are exceeded. In this study, subjects were divided into four groups, according to urinaryβ2-mg concentrations (1)<300 μg/ g cr.(2)300-<1000 μg/ g cr (3) 1000-<10000 μg/ g cr and (4) >10000 μg/ g cr. An association in male was demonstrated between mortality and urinary β2-mg concentrations in the two categories of >1000 ug/ g cr, compared with concentration of 300 μg/ g cr .In female, however, significant association were found in the three categories>300 μg/ g cr, compared with <300 μg/ g cr. The mortality rate tended to become higher as the severity of renal tubular dysfunction progressed [4] (Table 1). Accordingly, when evaluating the risk of Cd, a minimal objective should be to avoid increased excretion of such low molecular weight proteins. However, patients with itai-itai disease are officially recognized only in the Jinzu River basin, although the patients in other Cd-polluted regions showed the same clinical features and Cd concentration in the organ as those of the patient with itai- itai disease [2,3].

Furthermore, renal tubular damage which are observed in different Cd polluted regions is not officially recognized as the health effects caused by exposure to environmental Cd. Therefore, in Japan, if we want to discuss on the risk estimation of Cd in the general environment, it is urgent to solve the problem mentioned above.

In Japan, since approximately 50% of Cd intake is derived from rice, the establishment of tolerable limits must take into account indices of external exposure such as Cd concentration in rice and lifelong Cd ingestion as well as indices of internal exposure like urinary Cd concentration. We conducted epidemiological surveys in the Cd-polluted Jinzu River basin in Toyama Prefecture and Kakehashi River basin in Ishikawa Prefecture and

demonstrated the presence of a dose-response relationship in the inhabitants of both basins, based on which we reported tolerable limits for Cd concentrations in rice, lifelong Cd intake, and urinary Cd concentrations [5-12] (Fig. 1-4 Table2-7). The following tolerable limits were described: Cd concentration in rice: 0.05～0.2ppm, lifelong Cd intake: 1.5~2.0g, and urinary Cd concentration in men: 1.6～3.0 μg/g.cr., and women: 2.3～4.6. μg/g.cr. (Table 8).

Since there was virtually no difference between these values and those noted in non-Cd-polluted areas, it was surmised that the inhabitants of non-Cd-polluted areas may similarly be adversely affected by Cd exposure. We have observed a dose-response relationship between urinary Cd concentration and rates of positive renal tubular findings in 3 non-Cd-polluted areas in Japan.

In this study, blood and urine samples were collected from 2,753 subjects (1,105 men and 1,648 women) ages over 50 years old. Blood was analyzed for Cd and urine was analyzed for Cd, total protein, β2-mg and N-acetyl-β-D-glucosaminidasase (NAG). Multiple regression analysis and logistic regression analysis were performed to clarify the dose-effect and dose-response relationship between blood or urinary Cd concentration and indictors of renal dysfunction. Both analyses showed a significant relationship between Cd in blood and urine and indicators of renal dysfunction[13] (Table 9-12). In another study, urinary total protein, β2-mg , NAG and urinary Cd were measured using 2sets of 24-h urine samples from each of 829(411 men and 418 women)subjects aged 40-59 years in three areas without any known environmental Cd pollution. Using expressed units (g creatinine and day^{-1}) in the multiple regression analysis, the partial regression coefficients demonstrated a significant association between urinary Cd concentration and total protein,β2-mg and NAG in both sexes. The same results were obtained in both sexes in the logistic regression analysis. We are currently endeavoring to establish tolerable limits by applying new statistical methods to hitherto obtained epidemiological data and at the same time to better characterize the nature and significance of renal tubular dysfunction in the inhabitants of non-Cd-polluted areas (Table 13).

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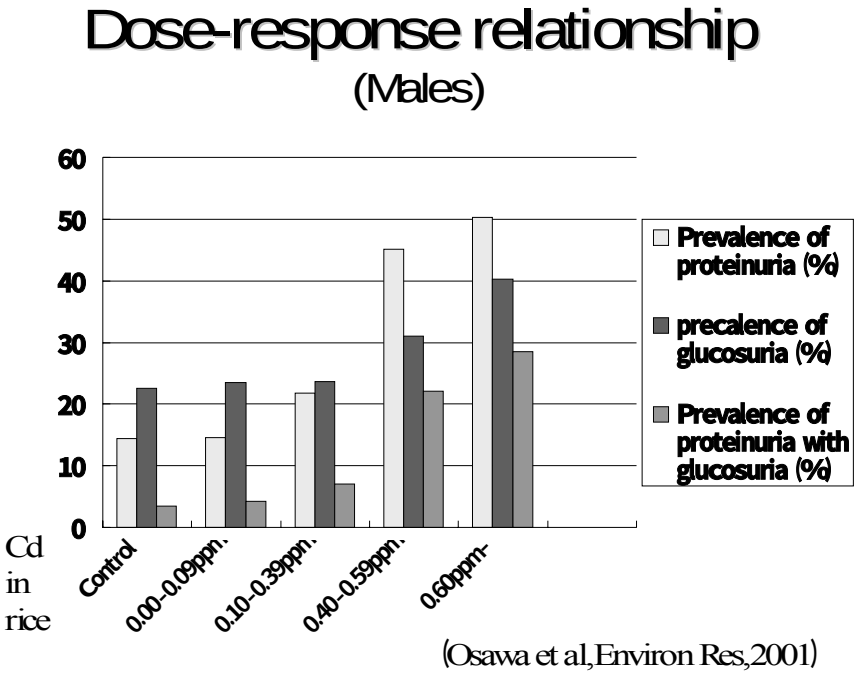
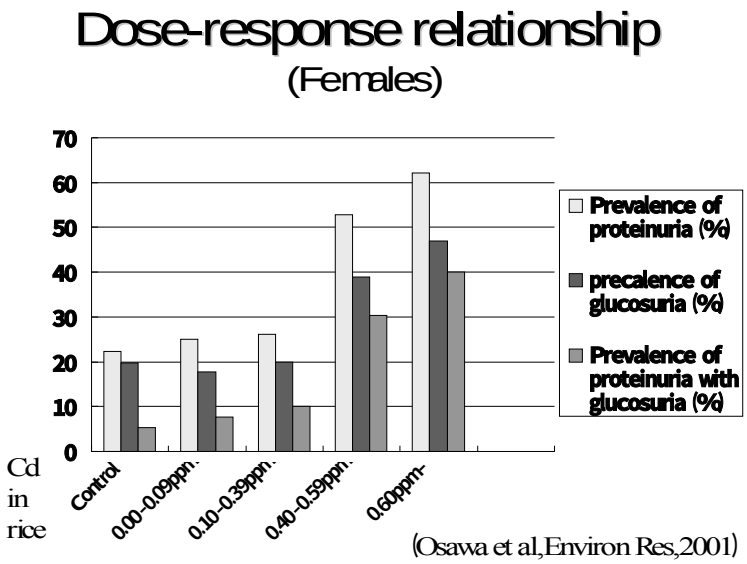
Fig. 1



(Osawa et al, Environ Res, 2001)

Fig. 2

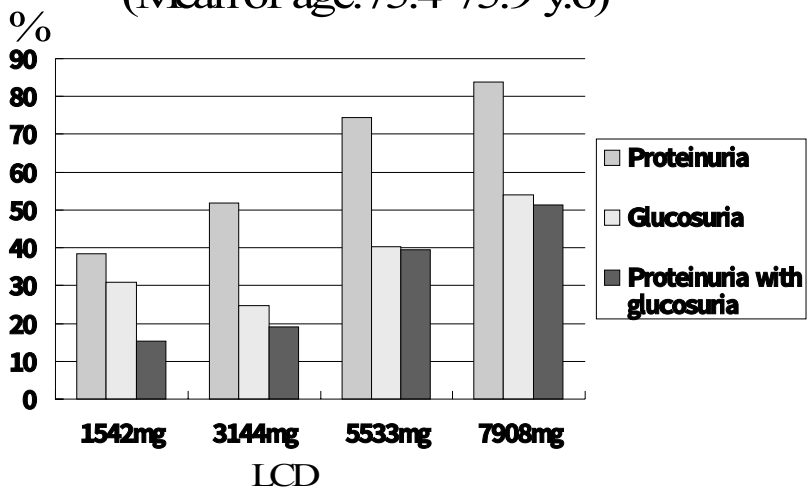


(Osawa et al, Environ Res, 2001)

Fig. 3

Relationship between life—time Cd intake and urinary abnormarity rates (Males)

(Mean of age: 75.4-75.9 y.o)

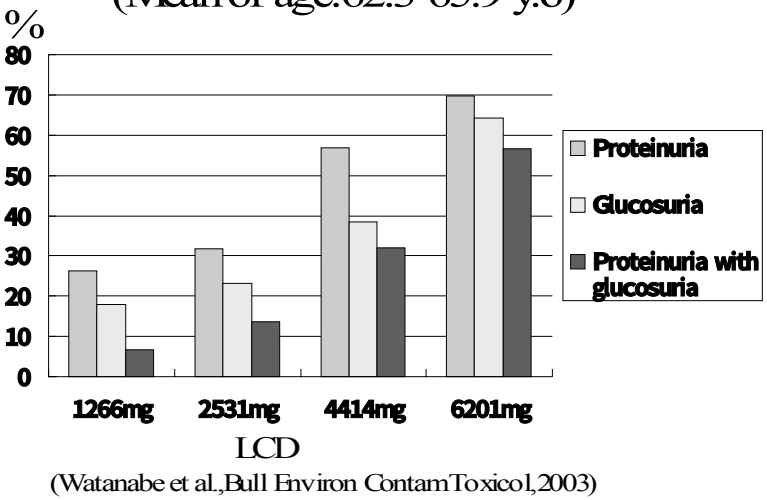


(Watanabe et al, Bull Environ Contam Toxicol, 2003)

Fig. 4

Relationship between life—time Cd intake and urinary abnormarity rates (Females)

(Mean of age: 62.3-65.9 y.o)



(Watanabe et al., Bull Environ Contam Toxicol, 2003)

Table 1

Mortality and β_2-microglobulin in urine

Males	Hazard ratio	
β_2-MG(1)	1.27 (1/0)	0: =<300 μg/g cr
β_2-MG(2)	1.47 * (2/0)	1: 300-<1000 μg/g cr
β_2-MG(3)	1.69 * (3/0)	2: >1000-<10000 μg/g cr
		3: >10000 μg/g cr
Females		
β_2-MG(1)	1.58 ** (1/0)	
β_2-MG(2)	2.04 *** (2/0)	
β_2-MG(3)	2.43 *** (3/0)	

(Nakagawa et al., Arch Environ Health, 1993)

Table 2

Relationship between Cd in rice and urinary abnormality rates

	Number of hamlets	Urinary findings	Correlation coefficients	Tolerable Cd in rice(ppm)
Males	56	Proteinuria	0.71	0.08
		Glucosuria	0.41	0.19
		Proteinuria with glucosuria	0.68	0.11
Females	61	Proteinuria	0.67	0.13
		Glucosuria	0.67	0.18
		Proteinuria with glucosuria	0.75	0.11

(Osawa et al., Environ Res, 2001)

Table 3

Relationship between Cd in rice and urinary abnormality rates in individual subjects

Criterion variable	Explanatory variable	Odds ratio
Male		
proteinuria	R-Cd	9.27***
glucosuria	R-Cd	1.32
proteinuria with glucosuria	R-Cd	13.1***
Female		
proteinuria	R-Cd	9.77***
glucosuria	R-Cd	2.10*
proteinuria with glucosuria	R-Cd	17.0***

(Watanabe et al, Toxicol,2002)

Table 4

Tolerable levels of Cd concentrations in rice (ppm)

	Proteinuria	Glucosuria	Proteinuria with glucosuria
Male			
40y.o	0.29		0.08
50y.o	0.13		0.15
60y.o			0.06
Female			
40y.o	0.36	0.78	0.23
50y.o	0.17	0.45	0.10
60y.o		0.13	

(Watanabe et al, Toxicol,2002)

Table 5

Relationship between life- time Cd intake and urinary abnormality rates

	Number of groups	Urinary findings	Correlation coefficients	Tolerable life- time Cd intake(mg)
Males	16	Proteinuria	0.84***	1324
		Glucosuria	0.82***	2697
		Proteinuria with glucosuria	0.89***	1473
Females	12	Proteinuria	0.94***	1527
		Glucosuria	0.88***	2175
		Proteinuria with glucosuria	0.92***	1570

(Watanabe et al, Bull Environ Contam Toxicol, 2003)

Table 6

Relationship between total-Cd intake and urinary abnormality rates in individual subjects

Criterion variable	Explanatory variable	Odds ratio
Male		
proteinuria	LCD	1.46***
glucosuria	LCD	1.05*
proteinuria with glucosuria	LCD	1.48***
Female		
proteinuria	LCD	1.45***
glucosuria	LCD	1.21**
proteinuria with glucosuria	LCD	1.61***

(Chiyoda et al, Biol Trace Element Res, 2003)

Table 7

Tolerable amounts of life-time Cd intake(mg)

	Proteinuria	Glucosuria	Proteinuria with glucosuria
Male			
40y.o	1.49	6.17	
50y.o	1.42	4.18	1.29
60y.o	1.39	2.15	1.57
70y.o	1.42	0.07	1.46
Female			
40y.o	1.60	2.56	1.25
50y.o	1.25	2.35	1.11
60y.o	0.94	2.17	1.00
70y.o	0.64	2.04	0.92

(Chiyoda et al, Biol Trace Element Res,2003)

Table 8

Tolerable level of cadmium

- Cd in rice: 0.08-0.11 ppm
- Life-time Cd intake: 1.47-1.57 g

(Based on the epidemiological studies in the Jinzu River basin)

Table 9

Relationship between Cd in urine and urinary abnormality rates in Cd-nonpolluted areas

(Multiple regression analysis)

Criterion variable	Explanatory variable	Standard partial regression coefficient	
Male	μg/g cr		MCC
protein	U-Cd	0.35***	0.37***
	Age	0.10***	
β 2-mg	U-Cd	0.14***	0.17***
	Age	0.09***	
NAG	U-Cd	0.12***	0.12***
	Age	----	

(Suwazono et al, Environ Res,2000)

Table 10

Relationship between Cd in urine and urinary abnormality rates in Cd-nonpolluted areas

(Multiple regression analysis)

Criterion variable	Explanatory variable	Standard partial regression coefficient	
Female	μg/g cr		MCC
protein	U-Cd	0.46***	0.44***
	Age	0.07**	
β 2-mg	U-Cd	0.19***	0.22***
	Age	1.11***	
NAG	U-Cd	0.18***	0.18***
	Age	----	

(Suwazono et al, Environ Res,2000)

Table 11

Relationship between Cd in urine and urinary abnormality rates in Cd-nonpolluted areas

(Logistic regression analysis)

Criterion variable	Explanatory variable	Odds ratio	Male μg/g cr
Male			
protein	U-Cd	3.92***	
	Age	1.04***	
β_2-mg	U-Cd	2.26***	
	Age	1.04***	
NAG	U-Cd	---	
	Age	1.02***	

((Suwazono et al, Environ Res,2000)

Table 12

Relationship between Cd in urine and urinary abnormality rates in Cd-nonpolluted areas

(Logistic regression analysis)

Criterion variable	Explanatory variable	Odds ratio	Female μg/g cr
Female			
protein	U-Cd	7.76***	
	Age	1.05***	
β_2-mg	U-Cd	2.84***	
	Age	1.05***	
NAG	U-Cd	1.88***	
	Age	1.05***	

((Suwazono et al, Environ Res,2000)

Table 13

Benchmark Dose Low_{10} of Urinary Cd

	Male	Female
	$g\,cr^{-1}$	
Protein	1.2	3.6
β_2-mg	0.7	1.3
NAG	0.6	1.2
	day^{-1}	
Protein	1.6	4.7
β_2-mg	1.1	1.6
NAG	0.8	0.5

[F-9]

RISK ASSESSMENT OF DIETARY CADMIUM EXPOSURE AND EXPOSURE ASSESSMENT IN JAPAN

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Backgournd Some recent reports suggest that environmental exposure to cadmium (Cd), even at low levels, may induce adverse effects in renal function and bone metabolism. To assess health effects due to low to moderate dietary Cd exposure close to the current Provisional Tolerable Weekly Intake (PTWI),

Methods Cross-sectional epidemiological studies were conducted among 1,960 female farmers from 8 districts in Japan. We collected peripheral blood and urine samples and medical and nutritional information, and we measured forearm bone mineral density by the dual energy X-ray absorption method. We also measured blood Cd (Cd-B) and luteinizing hormone levels, and urinary Cd (Cd-U), α_1- and β_2-microglobulin, and calcium levels, as well as four markers for bone metabolism. Dietary Cd exposure of the 8 populations was assessed from the individual Cd concentrations of the rice consumed by the study participants and the quantities of rice consumed daily. The populations showed a sequential difference in dietary Cd exposure, ranging from a level as low as that of the general Japanese population to one close to the current PTWI. In respect of renal dysfunction, the levels of urinary Cd excretion, an indicator of Cd accumulation in the kidneys, increased along the same sequential pattern as dietary Cd exposure. However, no differences were observed among the populations in levels of urinary α_1-microglobulin and β_2-microglobulin excretion, which are indicators of renal tubular function. In order to further confirm the contribution of aging to renal dysfunction, we analyzed the relationship between the levels of urinary proteinuria and age or Cd exposure levels in all subjects using the multiple regression models. taking each of the urinary proteins as a dependent variable and age, blood Cd (Cd-B)and urinary Cd (Cd-U) concentrations as independent variables. All of the models showed much bigger standard partial regression coefficients (SPRCs) for age than for Cd-B or Cd-U. Although the SPRCs of Cd-B or Cd-U were statistically significant ($p<0.05$) in the models due to the high degrees of freedom, their actual correlations with renal function were not considered to be significant since their partial correlation coefficients (PCCs) were very low. These results indicate that age is a much more important contributor to impairment of renal tubular function than Cd exposure at the levels observed in our study.

In respects of effects on bone, the populations of our studies are those exposed to Cd at a

level insufficient to induce kidney damage on bone mineral density and urinary calcium excretion

Findings Analysis of the data for subjects grouped by urinary cadmium level and age-related menstrual status suggested that cadmium accelerates the increase of urinary calcium excretion around the time of menopause and the subsequent decrease in bone density after menopause. However, multivariate analyses showed no significant contribution of cadmium to bone density or urinary calcium excretion, indicating that the results mentioned above were confounded by other factors.

Interpretation These results indicate that the current PTWI is sufficient to prevent Cd-induced renal dysfunction among the general population. Environmental exposure to cadmium at levels insufficient to induce renal dysfunction does not increase the risk of osteoporosis.

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Proceedings of
NIMD Forum 2005

平成１７年３月３１日発行

発行　環境省　国立水俣病総合研究センター
Published by National Institute for Minamata Disease

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March 2005

編集：安武　章、蜂谷紀之

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