CHAPTER IV

DISCUSSION & CONCLUSION

Cadmium is a toxic metal with a long biological half life, 10-30 years, in the human body[21]. The cadmium polluted area in Thailand was found in the Mae Sot district, in Tak province with a population of 12,075 in 12 villages. Paddy fields in the contaminated area were estimated to be approximately 13,200 rais[20]. It was reported that 85% of the determined rice grain taken from the area contained more cadmium than the recommended level of 0.2 mg/kg, and 85% of the determined paddy soil contained more than 3 mg/kg[20] Cadmium. During the years 2004-2005, Mae Sot General Hospital and Bureau of Occupational and Environmental Diseases, Ministry of Public Health did a health survey and found that 47.2% of the surveyed population had urinary cadmium between 2-5 µg/g Cr, and 7.2% had more than 5 µg/g Cr urinary cadmium. Renal dysfunction markers in the urine of the cadmium exposed population were also determined and found proteinuria with a high excresion of low molecular weight proteins. High excretions of calcium in the urine were also found with a decrease of the glomerular filtration rate[22, 23].

The number of Mae Sot inhabitants involved in this study was 700 people. Actually 705 inhabitants who attended the 2004-2005 health survey were selected to particitate, but one man and 4 women were excluded due to a shortage of the biological sample collection. Cadmium body burden was indicated by urinary and blood cadmium.

Cadmium exposure

Recent cadmium exposure and smoking influenced blood cadmium concentrations and it was found that blood cadmium increased within a few weeks after exposure started[85]. Using blood cadmium as an exposure indicator of the contaminated environmental cadmium should be treated cautiously in the case that if smoking continued, it could cause an overestimation of environmental cadmuim exposure.

Cadmium induced kidney damage causes an increased urinary excretion of cadmium, whereas when a decrease of urinary cadmium appears it might be due to losses of cadmium from the kidneys. In the case of concomitant tubular damage, the use of urinary cadmium as an estimated dose of cadmium exposure may thus be incorrect[85]. However, a study in Japan mentioned that even if renal dysfunction appeared, urinary cadmium could still be a good indicator of the environmental cadmium exposure, at least on a group basis[113]. The internal dose reference level set up by WHO was based on the concentrations of cadmium in the urine adjusted by a gram of creatinine and it was a good marker to indicate cadmium body burden.

Smoking also contributes to cadmium body burden[21]. One cigarette contains approximately 1-2 μg[72] cadmium concentrations. Among Japanese who live in cadmium polluted area, the smokers showed higher urinary cadmium than ex-smokers and non-smokers and mean of the urinary cadmium of the ex-smokers was higher than that of non-smokers[149]. In this study, non-smokers showed lower levels of blood and urinary cadmium than other smokers among men whereas urinary cadmium levels of non-smokers were higher than the urinary cadmium levels of ex-smokers and smokers in women (Table 7). This results show that smoking would not really be the

source of cadmium contributing to the cadmium accumulation in the studied women. Therefore, dietary consumption implied the main exposure route of cadmium among the studied women.

Cadmium absorption rate via inhalation was about 40-60% and it retained in blood circulation several weeks after exposure[72], on the other hand, cadmium absorption rate in the gastrointestinal tract was about 3-8% which was then sent to the liver, a production house of metallothionine[150]. Metallothionine forms a non-toxic complex with cadmium and this complex is circulated to kidney which is as accumulation site[3] of cadmium.

Age was a significant factor for an increase of cadmium body burden. Urinary cadmium level was gradually increased when age increased and it reached a peak at the age of 50-59 and then decreased again when age was over 60 (Figure 6). This result is in accordance to the previous finding that the average cadmium concentration in the kidney is near zero at birth, and rises up linearly with age to a peak between ages 50-60, after which cadmium concentrations in the kidney will plateau or decline[81]. Because of aging and postmenopause, inhabitants at the age of 50-59 were at risk to develop cadmium related pathologies, such as bone metabolic dysfunction, renal dysfunction and anemia.

We did not perform nutritional determination of the studied subjects to determine a relation between contaminated food and cadmium body burden. Only the amount of rice consumption was recorded by interview the subjects using rice model which approximately elucidated daily rice consumption among the subjects and it showed no relation to cadmium body burden. Simmons et al. (2005)[56] estimated the weekly cadmium intake value among the people who live in the polluted area by

determining concentrations of cadmium in rice and soil. The estimated weekly cadmium intake value range between 20-82 μg per kg body weight which was higher than the Provisional Tolerable Weekly Intake (PTWI) of cadmium at 7 μg per kg[56]. In order to determine relation between cadmium concentration in the rice or soil and internal exposure level, the cadmium concentrations in rice grain and soil samples from cadmium polluted area should be determined in the next health survey.

In conclusion, the factors contributed to an increase of cadmium body burden were aging, smoking, and farming. Urinary cadmium illustrated an accumulation of cadmium in the kidney whereas blood cadmium reflected more of recent exposure to cadmium. Because smoking contributed to cadmium exposure, a program to decrease smoking should be planned to diminish the cadmium exposure from cigarette smoke. Cadmium concentrations in rice and soil should also be monitored to verify external exposure among these inhabitants.

Bone metabolic abnormality

Bone formation and resorption are bone remodelling mechanisms of which it can be indicated by using osteoblast and osteoclast cells activity. Serum osteocalcin is a molecule produced by osteoblast and expressed during bone formation, whereas, urinary DPD and NTx are bone resorption markers produced by osteoclast from type I bone collagen[52, 131]. An increase of serum bone alkaline phosphatase and/or osteocalcin have been reported in Japanese whose urinary cadmium was ≥10 μg/g Cr accompanying with calciuria and low serum calcium[34, 36, 151]. In our subjects, even though serum osteocalcin was within a reference range of measurement[152], it was positively correlated to elevated levels of renal markers in women (Table 12). No

significant relationship between serum osteocalcin and urinary cadmium was observed in either gender after adjustment for age (Table 14). On the other hand, the relations between renal markers and serum osteocalcin were shown even after adjusting by mean age and urinary cadmium (Table 14). These findings suggest the increase in serum osteocalcin was probably due to renal dysfunction independent of age and cadmium concentration.

A hydroxypyridinium crosslinked collagen (DPD) is formed during the extracellular maturation of fibrillar collagens and is released in the degradation of mature collagens[134]. Measurement of DPD is not influenced by the degradation of newly generated collagens, or by dietary intake, and shows a high specificity for skeletal tissues. Type I collagen crosslinked N-telopeptide (NTx) and DPD have been identified as the best indicators for assessment of bone resorption[134]. In this study we found that urinary DPD and NTx were mainly related to an increase of urinary cadmium and/or renal tubule markers, suggesting bone resorption was related to cadmium body burden and/or renal tubule dysfunction in both genders (Tables 12-14). However, an inverse relationship between urinary DPD and β_2-MG was observed in women (Table 12). This finding suggests that urinary DPD might be decreased in women who have severe renal tubular dysfunction. The negative correlation between renal dysfunction and DPD was also shown by Coen et al. (2000)[153] and Aoshima et al. (2003)[36].

Urinary NTx, a metabolite of the N-terminal of mature collagen, was positively correlated with an increase of urinary cadmium and β_2-MG in both genders. This bone marker may be a more sensitive indicator of abnormal bone metabolism than DPD[134].

Urinary excretion of calcium was suggested as a useful indicator of renal tubular dysfunction by Ebeling and Akesson (2001)[131] and Seibel (2005)[52] and increased levels of urinary cadmium has been reported for inhabitants of cadmium polluted areas in Toyama, Japan[154] and in Belgium[15]. Wu et al. (2001)[17] has also reported a dose response relationship between urinary calcium and cadmium concentrations in Chinese who were exposed to cadmium. However, FECa reflects the calcium dynamics of renal tubular cells more precisely than urinary calcium[155]. In addition, Aoshima et al. (1993)[156] reported increased FECa with increased bone serum osteocalcin among Japanese with renal tubule dysfunction living in Toyama, suggesting urinary loss of calcium is an early indicator of osteomalasia caused by cadmium exposure.

By multivariate regression analysis of NTx, the independent variable set of age, U-Cd or B-Cd and FECa showed a higher coefficient of determination than that of a model containing NAG or β_2-MG in both genders (Table 13 and 14). These results showed more substantial relations between FECa and NTx than those of NAG and β_2-MG. Therefore, the FECa is a useful marker to determine the calcium wasting among cadmium exposed inhabitants and it should be further investigated to prevent bone resorption acceleration progression.

It has been reported that inhabitants of the Kakehashi River basin, Japan, had low serum calcium, accompanied by high levels of bone formation[40, 104]. In this study at Mae Sot, 43 men (27.6% of male subjects) and 73 women (28.5% of female subjects) had urinary cadmium concentrations ≥10 μg/g Cr (Table 10 and Table 11). These levels are similar to the highest levels recorded from Japan. However, the Mae Sot subjects still seem to be in the early stages of bone effect development. This may

be due to a shorter period of cadmium exposure and younger age of subjects compared to the Japanese cases.

In our study, the relations between bone remodelling markers and cadmium exposure indices were shown. The FECa was increased in high cadmium exposure group and it showed strong positive relation to bone resorption marker independently from age and cadmium exposure indices. Our result supported the hypothesis that the calcium handling imbalance and renal dysfunction caused by cadmium exposure are an explanatory factor of bone resorption acceleration among cadmium exposed inhabitants. Therefore, a follow up investigation of the urinary cadmium and calcium metabolism in all elderly persons living in the cadmium polluted area is highly recommended, to help reduce their suffering from renal and bone diseases.

Anemia prevalence

A relationship between cadmium exposure and anemia was previously reported[6, 108]. In animal models chronic cadmium exposure induced anemia by enhanced iron deficiency, and decreased erythropoietin production. These pathologies appeared together with proximal tubular dysfunction but conserved glomerular morphology [108]. An itai-itai patient showed severe proximal tubular dysfunction and a low level of erythropoietin with severe anemia but serum iron or ferritin was normal and it was normochromic normocytic anemia [6]. Glomerular dysfunction showed no relation to anemia or iron-deficiency among these patients. On the other hand, among non-polluted area inhabitants whose urinary cadmium is below 2 μg/g Cr, the relationship between anemia and cadmium exposure or proximal tubular dysfunction was not observed [45, 46].

Anemia prevalence among unexposed cadmium Thai urban population aged over 60 years were 18.50% in men and 13.00% in women [157], however, anemia prevalence in our studied inhabitants aged ≥60 years was 38.32% in men and 28.87% in women (Table 17) which was higher than previously reported.

A dose-response relationship between cadmium exposure and renal dysfunction was shown in Tables 10-11 indicating that cadmium induced both renal proximal tubules and glomerular dysfunctions. Dominantly, β_2-MG showed significant relation to urinary cadmium in both genders (Table 10-11) in accordance with previous finding of Teeyakasem et al. (2007)[23]. It confirmed that cadmium potentially enhanced irreversible proximal tubular dysfunction in these inhabitants. The prevalence of an increased cystatin C also indicated the glomerular dysfunction development in accordance to the report of Limpatanachote et al. (2009) [158]. Glomerular dysfunction prevalence among our subjects was more frequent than the subjects reported by Chittinandana et al. (2006)[159] in the Thai population who live in non-polluted area. Additionally, the glomerular dysfunction prevalence in Chinese residing in the cadmium polluted area whose urinary cadmium is over 5 μg/g Cr was only 9.35%[4].

Relation between anemia prevalence and proximal tubular or glomerular dysfunction were dominant in both men and women (Table 18 and Table 19) when analyzing the data with Chi-square test. However, in order to verify the relationship between anemia and urinary cadmium and renal dysfunction, the binary logistic regression model was used and after adjusting the relationship by age and U-Cd, increased anemia prevalence and renal dysfunction for both proximal tubular and glomerular segments were shown clearly in women (Table 20 and Table 21).

The expected odd ratio of the people with NAG >8 Unit/g Cr had wider 95% C.I. than the people with β_2-MG >1,000 μg/g Cr (NAG >8 Unit/g Cr Odds range: 1.05-10.26 vs β_2-MG >1,000 μg/g Cr odds: 1.56-4.92). This result elucidated that β_2-MG can help predict anemia prevalence more precisely than the NAG.

Dysfunction of proximal tubular cell occurred in the early stage of cadmium intoxication whereas the necrotic injury was shown in the later stage. In animal models anemia and a decrease of erythropoietin production were demonstrated only when proximal tubular cells injury appeared [109]. The evidence obtained from our study supported that when irreversible proximal tubular dysfunction developed, anemia prevalence increased.

Proximal tubular dysfunction was evidenced in iron-deficiency and β-thalassemia patients[160]. Anemia was proposed as a cause of proximal tubular dysfunction among these patients[161]. Proximal tubular cell contains numerous mitochondria indicating many physiological functions of the cell such as reabsorbing water, electrolytes and low-molecular-weight substances and hormone synthesis. In this context, it requires a lot of oxygen supply and it is sensitive to intracellular oxygen partial pressure [148]. Anemia could induce kidney hypoxia even under normal blood flow conditions [161].

Once that anemia developed, and it was not corrected, proximal tubular cell injury because of hypoxia could be shown. On the other hand, when proximal tubular cell dysfunction became severe it enhanced more severe anemia by lack of erythropoietin production. Consequently, the renal function such as essential nutrient reabsorption capacity, erythropoietin and vitamin D active metabolite synthesis was affected. Then, malnutrition, severe anemia and bone metabolic dysfunction

developed[21]. Therefore, anemia should be corrected among cadmium exposed inhabitant to prevent anemia complications and prevent severe kidney dysfunction.

The relationship between glomerular dysfunction and anemia is an unexpected result because glomerular dysfunction is not a dominant pathology of cadmium intoxication and it showed no relation to anemia in the previous study[6]. However, the relation between anemia and glomerular dysfunction was similar to those of chronic kidney disease patients who showed anemia when renal glomerular dysfunction became severe[43, 111].

Even renal dysfunction and cadmium exposure were proposed as a cause of anemia but studies of Japanese and Thais also showed that anemia could increase cadmium absorption[45, 82, 162]. Cadmium and iron are transported to blood circulation via Divalent Metal Transporter 1 (DMT1) and iron deficiency increased expression of these transportation proteins resulted in an increase cadmium absorption from the gastrointestinal tract[82]. Mean values of the urinary cadmium in anemia subjects were significantly higher than non-anemia group which supported the assumption that anemia increases cadmium accumulation. Therefore, to prevent an increase of cadmium accumulation, the anemia should be treated.

Normocytic normochromatic anemia was found in itai-itai patients which was shown by normal morphology of red blood cell but hemoglobin level was lower than the reference value[108]. In our study, MCV, MCH and MCHC in anemic women were lower than the reference levels (Table 15) which was a microcytic hypochromatic anemia. This result indicated abnormalities of both red blood cell morphology and hemoglobin level[147]. Low iron body burden and low erythropoietin were related to a decrease of hemoglobin in the cadmium exposed inhabitants[82]. Iron deficiency,

thalassemia, abnormal hemoglobin synthesis and chronic diseases were also causes of microcytic hypochromatic anemia[147]. Because high prevalence of β-thalassemia and α-thalassemia in Tak province were reported[163], these pathologies should be determined in the next study.

In conclusion, a relation between cadmium exposure and anemia was shown as well as the irreversible proximal tubular dysfunction and anemia. Glomerular dysfunction biomarker also positively related to an increase of anemia. Hemoglobin level should be determined continuously to follow up the severe anemia in the study subjects.

Cadmium exposure and genders

Most of the itai-itai patients were women and also pregnancy was proposed as a risk factor of itai-itai disease[5], therefore, it was concluded that women were more affected by cadmium than men[12]. Exposure level of cadmium in women was often found to be higher than men due to a difference in cadmium absorption, menstruation cycle and pregnancy[164]. However, in our study, urinary cadmium level in women was borderline significant higher than the urinary cadmium in men (6.97 vs 6.35 μg/ g Cr) whereas blood cadmium in men was higher than in women (6.71 vs 4.94 μg/l) (Table 8). High blood cadmium in men could be explained by high percentage of smokers of our population.

Severity of renal dysfunction in men was expectedly higher than in women due to higher β_2-MG and cystatin C levels in men than in women (Table 8) and higher prevalence of an increase of β_2-MG in men, 30.27%, (Table 18) vs 15.95% (Table 19) in women. This result was not in accordance with a previous study in which the

assumption was that renal dysfunction in women was more severe than men[114]. Other relevant factors such as diabetes and hypertension should be subjected to clarify renal dysfunction development among the inhabitants.

Bone pathology caused by cadmium exposure was shown dominantly more in women than men as illustrated in European countries[15, 38] and Japan[7, 34]. Women carried more bone pathology risk factors than men, therefore, exposed women should be concerned and cautiously follow up their bone formation and resorption to prevent or reduce severity of osteoporosis.

Anemia prevalence in women was increased with an increase of renal dysfunction severity even after adjusted by age and cadmium exposure level whereas in men cadmium level and renal dysfunction showed no relation to anemia (Table 20). A nutritional survey to determine anemia risk factors such as vitamin B deficiency, iron deficiency and malnutrition should also be additionally studied for an understanding of the mechanism of gender difference in cadmium intoxication.

Cadmium disturbs human physiological function over a wide range such as enhancing renal dysfunction, osteomalacia, osteoporosis, hypertension and anemia[21, 165, 166]. In order to study cadmium pathologies, biomarkers were used because of their high specificity and convenience using in large population scale[28, 167]. However, there are some limitations of using biomarkers, for example, DPD and NTx are bone resorption markers which were used to follow up bone dynamic but in our study NTx showed renal function dependence whereas an increase of DPD level related to the renal function (Table 12). Therefore, specific markers of cadmium-related pathology should be further identified. More advanced techniques will help to identify the lipid or protein metabolism involved such as multi-dimensional liquid chromatography,

matrix-assisted laser desorption/ionization (MALDI), two-dimensional gel electrophoresis, DNA microarray, protein microarray, mass spectrometry, and/or isotop-coded affinity tag[168-170].

Direct effect of cadmium on human fetal osteoblasts

Osteoporosis prevalence was found among the exposed inhabitants[35, 51] and caused bone resorption acceleration which was proposed as a cause of bone pathology, and it was also found in the section of the population with low levels of cadmium exposure[46, 49, 51]. The mechanism of this finding was proposed in 2 main aspects, the first one was an indirect effect which was shown after cadmium induced renal dysfunction[15] and the direct effect of cadmium to the osteoblast cells.

The direct mechanism of cadmium on bone cell was also evidenced[91, 93, 98, 171] on studying the cytotoxicity of $CdCl_2$ on the Saos-2 cells which was a human osteoblast- like cell line[98]. It was also found that cadmium directly disturbed calcium homeostasis and decreased collagen synthesis in rat osteosarcoma cells[91, 172]. This data showed that cadmium directly disturbed the function of osteoblast and increased osteoclastogenesis[173] which increased bone resorption. However, a relationship between osteoblast gene expression and cadmium exposure has never been reported.

Human fetal osteoblast like (hFOB 1.19) cell line expressed phenotypic characteristics of osteoblastic cells, including high alkaline phosphatase activity, 1,25-dihydroxy vitamin D3-inducible osteocalcin expression, and parathyroid hormone-inducible cAMP production. These cells display several advantages over primary culture human bone cells, in that they are more homogeneous, proliferate rapidly until they are forced to differentiate[174]. In this study, selected genes to show direct effect

of cadmium on hFOB cell were ALP, OC and Col1A1 which showed elevated in heavily cadmium exposed inhabitants[34, 36]. RANKL and OPG were selected to show the effect of cadmium exposure on osteoclastogenesis[58-60]. LC50 calculated from MTT assay resulting of the concentration of 23.17 μM (Figure 12) which was lower than those of MG-63[99] or Saos-2[98] cells. The morphology change was observed when $CdCl_2$ increased concentration to 10 μM (Figure 11) and calculated LC20 was 7.72 μM. Therefore, $CdCl_2$ concentration of 7.72 μM was used as the highest dose for gene expression experiments. We increased culture temperature to 37˚C for 2 days before treatment with a $CdCl_2$ concentration range 0.96-7.72 μM for 24 hr. At $CdCl_2$ concentration 7.72 μM, the OPG mRNA expression level was higher than that of dexamethasone treatment which was used as negative control of the OPG mRNA expression[55]. However, it showed no statistically significant between an increase of $CdCl_2$ concentrations and OPG mRNA expression level.

OPG and RANKL genes expression related to osteoclastogenesis in both *in vitro* and *in vivo*[54, 63]. In primary rat osteoblast cells, after 24 hr treatment with $CdCl_2$ RANKL gene expression increased more than the control and alkaline phosphatase increased[97]. However, our result did not support this assumption. $CdCl_2$ showed no relation to OPG mRNA expression (Figure 14) and RANKL mRNA expression could not be detected in our study which was similar to the previous study[55]. This variation might be due to osteoblast differentiation requires external activators such as vitamin D active metabolite, ascorbic acid and dexamethasone to obtain completely mature osteoblast and express osteoclastogenesis signal[175, 176].

Zhang et al. (2004)[177] reported that 1α,25-dihydroxy vitamin D_3 at concentration 10^{-8} M could up regulate RANKL mRNA expression in primary human

periodontal ligament cells after 6 days culture period in a time dependent manner. On the other hand, OPG mRNA expression was found in the absence of 1α,25-dihydroxy vitamin D_3 which was in accordance to our result that OPG expression was found in the culture condition without 1α,25-dihydroxy vitamin D_3 supplement. Another study also provided evidence that RANKL expression in osteoblast require a PTH signal[178, 179]. Additionally, in human chondrocyte, the expression of OPG and RANKL were enhanced by external cytokines, IL-1β, TNF-β and PGE_2[180]. This data implied that RANKL expression in osteoblast required an external signal and the time series effect should be determined. Therefore, in order to understand the direct mechanism of cadmium on bone cell, the differentiated factors such as 1α,25-dihydroxy vitamin D_3, ascorbic acid and dexamethasone should be supplemented to the culture condition and time series experiments should be performed.

Conclusion

Cadmium exposure among the study subjects was higher than the general Thai population and they also showed high levels of the biomarkers of renal dysfunction, bone resorption acceleration and anemia prevalence. Smoking and contaminated food consumption were the contributed factors for an increase of cadmium body burden. Increasing of calcium excretion played an important role to enhance bone resorption acceleration in the study subjects. Relation between anemia prevalence and proximal tubular and glomerular dysfunctions were shown in both men and women of the subjects. The follow up health program to determine exposure dynamics of cadmium will be advantage to prevent severity of cadmium intoxication, especially in women.

The *in vitro* results show effect of cadmium chloride on mRNA expression of the hFOB cells only with the osteocalcin, Col1A1 and OPG genes.