CHAPTER I

INTRODUCTION

1.1 Principle, Theory, and Rationale

Cadmium is a toxic metal which is mainly contaminated to environment via zinc mine and industrial processes[1]. Because of its long half-life in human body[2], cadmium could induce renal dysfunction[3], osteoporosis[4], osteomalacia[5] and anemia[6]. A dominant renal pathology induced by cadmium exposure is proximal tubular dysfunction which has been shown by low molecular weight proteinuria and a decrease of essential elements reabsorption [7]. The most severe cadmium intoxication is itai-itai disease and the patients have been reported with high cadmium body burden, anemia, severe proximal tubular dysfunction and multiple pseudofractures with severe bone pain[8].

Cadmium pollution

Cadmium pollution was discovered in Japan[9-11], Sweden[12, 13], Belgium[14, 15] and China[16-18] in which renal and bone metabolic dysfunctions were found among the exposed inhabitants. Recently, the cadmium polluted area in Mae Sot district, Tak Province, Thailand was disclosed. There were approximately 9,668 inhabitants with high risk of cadmium toxicities whose age was more than 15 years residing in the area[19]. A health survey conducted by the Mae Sot General Hospital and the Bureau of Occupational and Environmental Diseases, Ministry of Public Health in 2001-

2004[20] was found that 7.2% of the inhabitants had urinary cadmium more than 5 μg/g creatinine (Cr) which was 2.5 times of the recommended level of 2 μg/g Cr[20, 21]. Additionally, renal dysfunction was reported and shown to be related to cadmium exposure level[22, 23].

Cadmium induced renal dysfunction

Kidney is the accumulated site and target organ of cadmium[21]. When cadmium induced renal dysfunction, a decrease of vitamin D active metabolite, a decrease of erythropoietin synthesis, and an increase excretion of calcium (Ca) were shown[6, 12, 24]. Proximal tubular dysfunction is a dominant pathology of cadmium toxicity.[25] The proximal tubular cell has a specific lysozomal enzyme to degrade cadmium-protein complex which release free cadmium to cytoplasm. Free cadmium then disturbs cellular function such as electron transport, detoxifying molecule, and Ca metabolism[26, 27]. A function of proximal tubular cell is to reabsorb essential nutrient from glomerular filtrate[28]. When proximal tubular dysfunction occurs, an increase of low molecular weight proteins in the urine will be appeared and it could be used as specific proximal tubular dysfunction biomarkers. β_2-microglobulin (β_2-MG) and N-acetyl-β-D-glucosaminidase (NAG) were used in human study and they showed specificity to indicate proximal tubular dysfunction[11, 18, 29]. Excretion of β_2-MG >1,000 μg/g Cr was used to indicate irreversible proximal tubular dysfunction among inhabitants who lived in the polluted area[30].

Cadmium induced bone pathology

Previous studies[31, 32] indicated that cadmium firstly induced renal dysfunction and then enhanced bone pathology. Once the proximal tubular dysfunction appeared the Ca reabsorption capacity and vitamin D active metabolite, produced by proximal

tubular cells, were decreased[33-35]. A vitamin D active metabolite diminution caused an increase of bone resorption and bone pathology. Bone formation markers; serum osteocalcin (OC) and alkaline phosphatase, and bone resorption markers; urinary N-telopeptide (NTx) & C-telopeptide (CTx) of type I collagen and urinary deoxypyridinoline (DPD) were used to determine bone metabolic dysfunction in cadmium exposed inhabitants[36]. Positive relation between bone resorption markers and cadmium exposure levels were shown among low level cadmium exposed inhabitants[12, 37, 38]. An increase of bone formation marker was also shown among heavy cadmium exposed inhabitants and it showed positive relation to renal tubular dysfunction[39, 40].

The turnover of bone metabolism among cadmium exposed inhabitants was not normal, especially the bone resorption rate[37]. Renal dysfunction was assumed as a dominant cause of bone metabolic dysfunction. However, previous study in Japan[41] was proposed that renal tubular dysfunction alone might not be able to elucidate the cadmium induced bone pathology mechanism because there was no relation observed between cadmium induced renal dysfunction and bone pathology among the subjects exposed to cadmium at low level.

Therefore, the renal and bone pathologies including the relevant mechanisms of toxicity caused by cadmium exposure among Thai people in Mae Sot were investigated even though the pathologies might not be as severe as with the itai-itai patients in Japan. The study would help as an early cadmium-induced pathologies detection, in order to prevent the permanent pathological occurring among these high risk people.

Cadmium and anemia

Increasing of anemia prevalence among high cadmium exposure inhabitants has been reported[6] but the results still be controversy. Berns (2007)[42], McClellan et al. (2004)[43] and McFarland et al. (2008)[44] reported that anemia prevalence in chronic kidney disease was increased with severe glomerular dysfunction, whereas Moriguchi et al.(2003)[45] and Tsukahara et al. (2003)[46] reported that environmental cadmium exposed inhabitants showed no relation between cadmium level and anemia or iron deficiency. However, itai-itai patients showed severe anemia accompanying with proximal tubular dysfunction[6]. A decrease of serum erythropoietin was proposed as a cause of anemia and it was found after proximal tubular dysfunction appeared.

The glomerular dysfunction among Thai exposed inhabitants has also been illustrated[22] but it's relation to anemia has not yet been investigated. Therefore, in order to prevent severe anemia occurrence in Mae Sot people, an association between anemia and proximal tubular and/or glomerular dysfunctions were determined.

Cadmium toxicities and in vitro model

Cadmium was evidently accumulated in bone tissue and possibly disturbed bone cell function[47]. Human fetal osteoblast like cell (hFOB 1.19) has ability to differentiate into mature osteoblast expressing the normal osteoblast phenotype. It provides a homogenous, rapidly proliferating and differentiating osteoblast physiology[48-51].

Alkaline phosphatase (ALP), collagen 1A1 (Col1A1), and osteocalcin (OC) have been reported as the osteoblast synthesis proteins to produce osteoid[52, 53]. They were specific to produce bone matrix and deposit calcium to the matrix.

Osteoprotegerin (OPG) and receptor activator for nuclear factor kappa B ligand (RANKL) were also investigated as the signal-molecules produced by osteoblast to enhance osteoclast formation[53-55].

Abnormal bone tissue formation which shown by high level of serum OC and ALP, and an increase of bone resorption were observed among cadmium exposed inhabitants[12, 36, 37, 39]. In order to verify the direct effect of cadmium on bone cell physiology, an *in vitro* experiment was performed to investigate the expression of the related bone cell's genes.

In this project, analytical experiments and epidemiological study were planned and conducted to determine cadmium induced bone metabolic dysfunction. The prevalence of anemia among cadmium exposed inhabitants was investigated. Low molecular weight proteins and proximal tubule lysozomal enzyme were used as the proximal tubular cell injury markers whereas serum cystatin C which has been reported as a newly developed marker for indicating glomerular dysfunction was also analyzed. A population characteristic such as age, sex, occupation, drinking and smoking habits, amount of rice consumption and individual diseases of the studied subjects were interviewed via questionaire to determine the relation of bone metabolic and renal functions caused by environmental cadmium exposure. Direct effect of cadmium on bone cell's genes expression was also investigated. Osteoblast specific genes and bone resorption related genes were used to elucidate gene expression profile after exposure to cadmium.

Literature review

1.2 Cadmium polluted area in Thailand

High level of cadmium contaminated in the environment in Mae Sot district, Tak province was first reported and pulled public concern[56]. Population number of Mae Sot district was 71,427 (36,855 men and 34,572 women) and the population in 3 cadmium polluted subdisticts; Mae Ku, Mae Toa and Phrathat Pha Daeng, was 8,557 men and 8,004 women[57]. The scientific finding of cadmium in soil in this area was higher than an international recommended level at 2 mg/kg and other area of Thailand[56, 58]. More than 90% of the rice grain contained cadmium higher than 0.2 mg/kg. The inhabitants are at risk to cadmium exposure via consumption of rice and vegetable grown in this area. Twenty five percents of these inhabitants had urinary cadmium over 2 μg/g Cr [20].

Zinc mines in Mae Sot district were started around 1977 [19] and owned by different owners during processing. They were claimed to be a cadmium contamination sources. One zinc mine was abandoned whereas another big zinc mine still be processing. Cadmium contaminated to the central water irrigation system which was used as a water source for farming and daily activity.

Farmer is Mae Sot's people's main occupation. They grow rice in their paddy field and use the water from the central water irrigation system for daily life. The government tried to stop growing rice in the cadmium polluted area to prevent further exposure and also distribution of cadmium contaminated rice to other communities[19].

In 2004, the urinary cadmium in these inhabitants was measured as an index of body burden by Mae Sot General Hospital team and Bureau of Occupational and Environmental Diseases, Ministry of Public Health. The target population was 9,668

inhabitants whose age was equal or more than 15 year old from 12 villages in this area and 7,697 (79.6%) inhabitants participated. It was reported that, 377 (4.9%) subjects showed 5-10 μg/g Cr of urinary cadmium, whereas 177 (2.3%) subjects had urinary cadmium ≥ 10 μg/g Cr[20]. An increased prevalence of renal dysfunction including irreversible proximal tubular dysfunction was evidenced[22, 23].

Limpatanachote et al (2007).[22] also determined cadmium induced pathology among Thai exposed inhabitants. They showed that urinary β_2-MG level was increased with an increase of urinary cadmium levels. Both of an increase of serum creatinine and a decrease of glomerular filtration rate (GFR) appeared to be associated with an increase of urinary cadmium. They concluded that excessive exposure to cadmium produced renal dysfunction among Mae Sot population.

Teeyakasem et al. (2007)[23] also showed that urinary NAG positively correlated with urinary cadmium in both male and female subjects with and without diseases such as hypertension, diabetes, nephropathy, osteopathy, urinary calculi, bone fractures, anemia, and hypertrophy of the prostate. The prevalence rates of urinary NAG excretion above 8 units/g Cr increased with cadmium exposure in a dose dependent manner among subjects with diseases. In contrast, increased prevalence of β_2-MG above 0.4 mg/g Cr was associated with cadmium above 5 μg/g Cr only in those without diseases. They concluded that urinary β_2-MG and NAG should be used together with urinary cadmium in the monitoring of renal toxicity in a population exposed to high-level cadmium coupled with high prevalence of chronic diseases.

1.3 Cadmium pollution in Japan and Itai-itai disease

Itai itai disease is a severe cadmium poisoning with bone damage, renal dysfunction and severe anemia[59]. The endemic of itai-itai was found in Toyama prefecture of Japan[60]. In the endemic area, cadmium had been transported to a big river, Jinzu river, by the Kamioka mine. The Kamioka mine, located just beside the Jinzu River about 55 km south of Toyama City, had been established about 400 years ago[59].

Itai-itai characteristics were summarized by Nogawa and Kido (1996)[59]. Most of the patients were postmenopausal women. Severe bone pain all over the body and a duck-like gait were characteristic of the disease. These conditions continue for several years, after which patients were finally confined to bed. The slightest external pressure could cause bone fractures, such as coughing. Patient couldn't sleep and respiratory movement was restricted because of the severe pain. Body height was reduced from the normal level up to 30 cm in severe cases[59].

Blood examinations among this patient showed a decrease of inorganic phosphorus in serum together with an increase alkaline phosphatase. Normal or low-normal range of serum Ca levels was observed. Hemoglobin value and erythrocyte counts values were decreased in the itai-itai patients[59].

Urinary findings showed that itai-itai patients had increase excretions of total protein, glucose, animo-N, proline, Ca, low molecular weight protein. The Ca/P ratio in urine was highest in the patients with itai-itai disease. Urinary excretion of Cd in itai-itai patient ranged 10-30 μg/g Cr[59].

Bone radiological findings of itai-itai patients were osteomalacia and marked decalcification. Looser's zones (a narrow radiolucency which transects one or both

cortical margins of a bone and is a certain indication of osteomalacia) were shown. Deformities of the frame were frequently seen in pelvic bone, costae, and thoracic and lumbar vertebrae[61].

Pathological findings showed that the principal changes were observed in bones and kidneys. Osteomalacia was also confirmed by bone biopsies together with osteitis fibrosa. Even the kidneys were highly contracted but there were no obvious changes in the glomeruli. Tubule pathology consisted of marked atrophy, degeneration and dilation. Liver cadmium concentrations were in the range of 63-132 μg/g wet weight[59].

The patients were suffered by severe bone pain and women are 90% of all the patients, children number and nutritional status were the co-risk factors for this disease. It was clearly shown that the bone damage was developed by cadmium induced renal tubular dysfunction and the renal tubular dysfunction in itai-itai patient was irreversible[5]. The renal tubular dysfunction illustrated by low molecular weight proteinuria, glucosuria, amino acid uria, calciuria[62], includingly, the glomerular function impairment showing by an increase of serum creatinine[61].

After the progression of renal tubular dysfunction appeared the vitamin D active metabolite production impaired, parathyroid hormone (PTH) level increased, the reabsorption of Ca and phosphorus (P) from gastrointestinal tract decreased with Ca excretion increased resulting of bone damage developed[33, 63]. The cadmium exposure level among itai-itai patient was higher than 30 μg/g Cr which could be categorized as extremely heavy exposure level and it was typically found among Jinzu river basin inhabitants[62, 64].

Nogawa et al. (1996)[65] and Ogawa et al. (2004)[60] reported that the prevalence of itai-itai patients was increased as cadmium concentrations in rice increased.

Honda et al. (2003)[41] determined possible kidney damage and bone mass loss in non-cadmium-polluted area women aged 40-88 years. The exposure mean was 2.87 μg/g Cr and it showed no correlation to proximal tubular dysfunction marker. Bone loss associated with increase urinary cadmium independently from cadmium-induced kidney damage. A two-fold increase in urinary cadmium was accompanied by a decrease in bone density corresponding to a 1.7 year rise in age. Horiguchi et al. (2005)[32] reported that female farmers who consumed rice contaminated by low-to-moderate level of cadmium had an increase risk of osteoporosis when renal dysfunction shown.

Omote et al. (2006)[37] determined urinary N-terminal cross link of type I collagen (NTx) level in cadmium-exposed Japanese aged over 50 years old used to reside and found positive significant relation between NTx and urinary cadmium. All the women were at risk to develop bone pathology.

1.4 Cadmium pollution in other countries

Nordberg et al. (2002)[66] reported that cadmium concentration in rice grain highly polluted area in China was 2.4 mg/kg. The Chineses showed statistically significant dose-response relationships between urinary cadmium or blood cadmium and renal dysfunction.

Wang et al. (2003)[16] reported that the prevalence of osteoporosis in women increased from 34.0% in the control area to 51.9% in the cadmium heavily polluted

area, among subjects over 50 years old. They concluded that environmental exposure to cadmium is associated with loss of bone mineral density in both males and females, leading to osteoporosis and increased risk of fractures, especially in the elderly and in females.

Jin et al. (2004)[4] determined a possible relationship between cadmium nephropathy and its effects on the skeleton and showed that proximal tubular dysfunction increased osteoporosis prevalence whereas glomerular dysfunction did not.

Åkesson et al. (2006)[12] found negative effect of low-level cadmium exposure on bone among Swedish women age 53-64 years old who environmentally exposed to cadmium with mean urinary cadmium was 0.67 μg/g Cr. Another study was done among Swedish including both men and women and showed fracture risk was absent before age of 50 whereas after the age of 50, the fracture hazard ratio increased by 18% per unit urinary cadmium (nmol Cd/mmol Cr)[67]. Järup et al. (1998)[68] studied the possible role of cadmium as a risk factor for osteoporosis by determining bone mineral density (BMD) of workers exposed to cadmium at a plant that manufactured heat exchangers and coolers. The results showed that workers with tubular proteinuria had lower forearm BMD than workers without tubular proteinuria.

In Belgium, Staessen et al. (1991)[69] reported that in postmenopausal women, a two-fold increase in urinary cadmium correlated with 0.01 g/cm^2 decrease in bone density. The relative risks associated with doubled urinary cadmium were 1.73 for fractures in women. Schutte et al. (2008)[38] found that in the absence of renal tubular dysfunction, environmental exposure to cadmium increased bone resorption in

women, suggesting a direct osteotoxic effect with increased calciuria and reactive changes in calciotropic hormones.

1.5 Environmental cadmium distribution to human

Cadmium is a rare metallic element, and falls in group IIB of the Periodic classification between zinc and mercury[70]. Human exposes to cadmium via rock-soil/water-plant-animal pathway. Greenockite (CdS) is a species in igneous rocks and ore which is transformed to $Cd_2(PO_4)_3$, $CdCO_3$, CdSe, CdO, $CdSO_4$, $CdCl_2$, inorganic or organic conjuagted form by metallurgical industry and mine process[71, 72]. These cadmium species then pollute to the environment.

Cadmium can exist as different chemical species in natural water and soil such as hydrated ions, iorganic and organic ligand conjugated complex, chloro, carborato and hydroxo complexes[70]. In natural water resource it mostly exists in free ion (Cd^{2+}) and organic conjugate complexes such as amino, fulvic, humic, nucleic acid, proteins, and thioamines complexes[73, 74]. Cadmium can be adsorbed to the soil component with higher selectivity coeffiecients for specific adsorption reaction than calcium (Pb>Cu>Zn>Cd>Ca)[71]. Cadmium complex is exchangeable between soluble and insoluble form. Free ion and conjugated cadmium complex are soluble cadmium species which are available for plant uptake[70].

Plant uptake cadmium by exchange adsorption, non-metabolic irreversible binding to sites within the cell wall, symplastic movement and simple diffusion[71]. Cadmium uptake rate in plant ranges 0.4-7.0% causing cadmium accumulation in root, leaf, grain, stem and flower[71]. The accumulation of cadmium in tobacco leaf and rice grain is a main source of environmentally cadmium exposure[75].

Once that cadmium in plant is transported to human body by both ingestion and inhalation, it is absorbed to blood circulation and forms complex to various kind of protein such as albumin, glutathione, cysteine contained protein and metallothionine[72]. Divalent metal transporter 1 (DMT1) was proposed as a key transporter of enterocyte cadmium uptake[72]. Organic anion and/or amino acid transporter, organic cation transporter, metal transport protein 1, and inorganic anion exchanger also contribute to cadmium uptake in enterocyte, hepatocyte and renal cells [72, 76-78]. However, endocytosis and simple diffusion were also proposed as a cadmium transportation[72].

After cadmium-complexes uptaken by enterocyte and transported to hepatic cell, the complex is degraded releasing Cd^{2+} free ion to cytoplasm by specific lysosomal enzyme[27]. The Cd^{2+} free ion induces transcription of metallothionine which binds to cadmium with high affinity[72]. Then Cd-metallothionine complex is circulated to kidneys which are accumulated site and target organ of cadmium[21]. When the accumulated cadmium concentration in kidneys reaches critical concentration, proximal tubular dysfunction is developed and it is a cause of cadmium related pathology such as osteomalacia, anemia, and hypertension[3].

1.6 Cadmium toxicity

Cadmium is a toxic metal contaminated to the environment via human activities such as zinc mine, industrial process and garbage burning[38, 79]. In the environment, cadmium exists in only one oxidation state (+2) and does not undergo oxidation-reduction reactions[79]. This toxic metal is exposed to human via food consumption, tobacco smoke or polluted air inhalations[80]. The inhaled cadmium will

be absorbed to the circulation 10-50% base on it's particle size whereas 3-8% of ingested cadmium is absorbed via gastrointestinal (GI) tract [79].

The content of cadmium is often 1-2 μg per cigarette. It has been estimated that smoking of 20 cigarettes per day results in a daily inhalation of 2-4 μg cadmium[3]. The inhaled cadmium will be transported to the circulation via pulmonary vein and derived to liver mainly via hepatic artery. On the other hand, ingested cadmium is absorbed in the GI tract and sent to the liver via superior mesenteric vein. During circulates in a blood stream, cadmium binds to albumin, cysteine containing protein and glutathione (GSH).

High ingestion level of soluble cadmium-salts causes acute gastroenteritis. Long-term occupational exposure to cadmium has caused severe chronic effects, predominantly in the lung and kidney. Chronic renal effects have also been seen among the general population. The accumulation of cadmium in the renal cortex caused a renal tubular dysfunction with impaired reabsorption of, for instance, proteins, glucose, and amino acids. A characteristic sign of tubular dysfunction is an increased excretion of low molecular weight proteins in urine. In some cases, the glomerular filtration rate decreases. Increase in urinary cadmium correlates with low molecular weight proteinuria, and the absence of acute exposure to cadmium may serve as an indicator of renal effect[1]. In more severe cases there is a combination of tubular and glomerular effects, with an increase in blood creatinine in some cases. For most workers and people in the general environment, cadmium-induced proteinuria is irreversible.

Urinary excretion of cadmium is related to body burden and renal damage. In people with low exposure level, the urinary cadmium level is mainly related to the

body burden. cadmium-exposed people with proteinuria generally have higher cadmium excretion than those people without proteinuria. After high exposure ceases, the urinary cadmium level will decrease even though renal damage persists. The interpretation of urinary cadmium is thus dependent on exposure period, exposure concentration, smoking status, age and renal dysfunction[64, 81, 82].

High exposure to cadmium may lead to the development of osteoporosis and/or osteomalacia[68]. There was evidence that long-term occupational exposure to cadmium might contribute to the development of lung cancer, but observations from exposed workers had been difficult to interpret because of confounding factors[83]. For prostatic cancer, evidence to date was inconclusive, but it did not support the suggestion from earlier studies of a causal relationship. At present, there is no convincing evidence for cadmium being an etiological agent of essential hypertension. Most data spoke against a blood pressure increase owing to cadmium, and there was no evidence of an increased mortality rate due to cardiovascular or cerebrovascular disease. Data from studies on groups of occupationally exposed workers and groups exposed in the general environment showed that there was a relationship between exposure levels, exposure durations, and the prevalence of renal effects[81].

Acute Toxicity (oral exposures)

Doses of 20 to 30 mg/kg of cadmium have resulted in human fatalities, but generally, fatal poisoning from cadmium is rare. High doses of cadmium are known to cause gastrointestinal irritation resulting in vomiting, abdominal pain, and diarrhea[84]. Following ingestion of cadmium, an asymptomatic period of 0.5 to 1.0 hour may precede the onset of clinical signs. Depending on the severity of exposure, clinical

signs of cadmium poisoning following acute exposure include: nausea, vomiting, abdominal cramps, headache, muscle cramps, exhaustion, shock, and death[81].

Subchronic Toxicity

Because the toxic effects of cadmium are functioned by a critical concentration being attained in a target organ, similar effects will occur following long-term exposure to low cadmium levels and short-term exposure to high concentrations[85]. Consequently, renal and hepatic toxicity may occur if toxic cadmium levels are attained in these organs even during subchronic exposure. A description of cadmium-induced toxicity following oral exposure is presented in the following section.

Chronic Toxicity

Chronic intoxication is associated irreversible renal failure, bone disorders, anemia and immuno-suppression[86]. The renal tubular dysfunction and bone metabolic dysfunction such as osteopenia and osteomalacia are dominant pathologies caused by cadmium exposure[39]. The most severe cadmium intoxication victim is itai-itai disease patient who showed both bone pathology and renal tubular dysfunctions. Urinary cadmium level among these patients ranged 10-30 μg/g Cr. On the other hand, even the exposure level was not as high as in those of itai-itai patients but bone resorption acceleration was shown among the environmental exposure inhabitants[12, 38].

1.7 Cellular toxicity of cadmium

The basic cadmium toxicity is its negative influence on enzymatic system of cells, resulting from substitution of other metal ions (Fe3+, Cu2+ and mainly Zn2+ and Ca2+) in metalloenzymes and it has very strong affinity to biological structure containing –SH groups, such as proteins, enzymes and nucleic acids [(87)].

Cadmium has many similarities to Zn ion such as two electrons configuration on the outer shell. These metals are linked to macromolecules, primarily through sulphur, oxygen and nitrogren. Cadmium affinity to SH-ligands as well as to N-donors is greater than that of Zn[(87)]. Even Ca is not transition metal and share an electron configuration like cadmium but its ion has the same oxidation number like cadmium ion. Ionic radius of Ca2+ (1.00 Å) is similar to that of Cd2+ (0.95 Å)[(27)]. Therefore, the toxicity or bioavailability of cadmium in living organism involve the physiological function of Ca and Zn such as mimics Ca ion in transport system via Ca channel, competes Zn to bind metallothionine protein, disturbs zinc containing enzyme and may decrease the accumulation of these 2 essential metals in the cell[(27, 88)]

The molecular toxicity of cadmium was based on the detoxification molecule diminution because cadmium itself cannot generate free radical but it can bind to sulfhydryl containing molecule such as glutathione, metallothionine and cysteine rich protein[(72)]. Cellular injury induced by cadmium exposure involves dissipation of the mitochondrial transmembrane potential, respiratory dysfunction and initial increases of the generation of reactive oxygen species (ROS)[(48, 50)]. The intracellular concentration of Ca which is a universal and versatile signal messenger is affected by cadmium. Cadmium can up-regulate the internal Ca concentration disturbing transduction pathways with mitotic or pro-apoptotic consequences[(88)].

At low treated level, cadmium showed damaging effect on cadherin junction in proximal tubule cell. The cadherin is a transmembrane Ca(II)-binding glycoprotein playing important role in cell-cell adhesion. It can bind cadmium to Ca(II)-binding regions, changing the glycoprotein conformation which then change epithelial barrier function, transport process and finally promote cellular toxicity[89, 90]. Additionally, cadmium affects the expression signal transduction molecule such as protein kinase C which is the Ca homeostasis regulating molecule [91], Immediate early response genes (IEGs) such as *c-fos, cjun and c-myc* which are protooncogenes controlling cell growth and division[90]. By these mechanisms cadmium induces different cellular toxicity depends on the target cell and exposure level.

Anke et al. 1998[92] found an increase of cadmium accumulation in bone tissue of inhabitants who lived in heavily cadmium polluted area. Abnormal synthesis of bone tissue indicated by an increase of bone formation markers; serum osteocalcin[34, 36, 39] and bone alkaline phosphate[40]. Additionally, bone resorption-acceleration indicated by high level of deoxypyridioline (DPD), N-terminal and C-terminal crosslink of type I collagen (NTx and CTx) was evidenced even in low cadmium exposed inhabitants[12, 37, 38]. In addition, the bone resorption acceleration and abnormal bone tissue synthesis caused by cadmium exposure was shown to be independent to kidney dysfunction[93].

The bone remodeling is a function of osteoblast, osteoclast and osteocyte. Osteoblast plays an important role to synthesize a bone tissue, then, differentiates to be osteocyte supporting a bone tissue. Osteoblast cell also produces bone resorption signal to enhance osteoclastogenesis[94, 95]. A specific bone matrix component; such as

OC, ALP and collagen type I are synthesized by osteoblast to form healthy bone tissue[94]. Expression of these genes indicates a normal function of osteoblast.

The osteoclast has a responsibility to resorp bone, releasing Ca and P to the circulation according to the external signal. The increase osteoclast activity was shown, showing by increase bone resorption marker level among cadmium exposed inhabitants[37]. An osteoclastogenesis is enhanced by local factors released from nearby cells, including osteoblast-lineage cells, and the cytokines from other cells such as IL-11, prostaglandin E2, PTHrP and oncostatin M. These activators work together with osteoblast by stimulate osteoblastic expression of receptor activator of NF-B ligand (RANKL), the key regulatory molecule required for osteoclastogenesis. RANKL interacts with a receptor (RANK) expressed on the cell surface of mononuclear hemopoietic osteoclast precursors to trigger osteoclast formation[95]. OPG is also synthesized by osteoblast and it competes with RANKL to the receptor[96]. An increase OPG production decreased osteoclast formation and diminish bone resorption[54]. In primary osteoblast cell culture, RANKL gene was up-regulated by cadmium exposure whereas OPG gene did not[97].

At cellular level, cadmium could disturb function of the bone cell and induced cell death[98, 99]. Lévesque et al. (2008)[99] indicated that cadmium accumulation in human osteoblast cells at dose-dependent manner with LC_{50} value of 22 ± 2 μM $CdCl_2$. Cadmium also induced apoptosis via an increase of cytochrome C release from mitochondria and caspase 3[100-102].

1.8 Cadmium osteotoxicity

The osteotoxicity induced by cadmium has been proposed by 2 mechanisms, the direct effect on bone cell and indirect effect via renal tubular dysfunction[39, 98, 99]. The direct effect was cadmium disturbed signal transduction in osteosarcoma cell causing a decrease of collagen synthesis[91] in addition to accelerated differentiation of new osteoclast and activated activity of mature osteoclasts inducing bone resorption[103]. The indirect mechanism was shown by the association between renal tubular dysfunction and impairment of Ca metabolism[7].

Kido et al. (1989)[31] proposed three mechanisms of cadmium induced bone disease. The first mechanism was cadmium decreased vitamin D active metabolite production by enhancing renal cell dysfunction. Vitamin D active metabolite deficiency leaded to a reduced incorporation of Ca in bone. The second mechanism was cadmium decreased gastrointestinal Ca absorption, which, in turn, leaded to a reduced bioavailability of Ca in the body and might lead to bone decalcification. The third mechanism was that cadmium directly affected bone collagen metabolism.

Kido et al. (1991)[39] used serum osteocalcin and serum alkaline phosphatase to indicate bone metabolic rate among cadmium exposed-inhabitants who showed renal tubular dysfunction. They found that osteocalcin level was related to cadmium-induced osteopenia and glomerular dysfunction was not observed. They also found a significant relation between renal dysfunction and PTH or $1\alpha,25(OH)_2D$.

Vitamin D active metabolite level and PTH were determined among cadmium exposed inhabitants who showed renal dysfunction. A vitamin D metabolizing enzyme system in proximal tubule was disturbed which caused a decrease vitamin D

active metabolite, 1α,25$(OH)_2$D, production. A decrease 1α,25$(OH)_2$D level was closely correlated to PTH and β_2-MG levels[24, 33, 104].

Tsuritani et al. (1992)[105] found that a renal damage due to cadmium exposure leaded to the decreased in the serum 1α,25$(OH)_2$D level and increased in serum PTH level, and women were more vulnerable to cadmium-induced bone injury than men.

1.9 Cadmium and calcium metabolism impairment

Calcium reabsorption impairment showed significant contribution to explain bone pathology development among cadmium polluted area inhabitants[12]. The urinary Ca alone or in combination with fractional excretion of calcium (FECa (%)) was used to indicate Ca metabolism. It was proposed that Ca reabsorption impairment could be shown after proximal tubular dysfunction developed[17, 106].

Hayashi et al. (2003)[63] clarified a significance of urinary Ca excretion levels in bone damage. Subjects of this study were Kakehashi River basin inhabitants who environmentally exposed to cadmium. They showed positive relations between Ca and urinary cadmium and β_2-MG in both genders. They concluded that bone damage was not caused by increased excretion of Ca alone because urinary excretion levels of Ca did not differ greatly between people with and without bone damage.

Kido et al. (1993)[106] investigated renal handling of Ca in a population with renal dysfunction induced by environmental cadmium exposure. Clearance method was used to determine reabsorption capacity of Ca. They showed that urinary excretion rate of Ca tended to be lower in the cadmium-exposed subjects than in the non-exposed subjects. The urinary excretion rate of Ca was closely related to creatinine clearance. They concluded that in cadmium-induced renal dysfunction the

urinary excretion of Ca depends on glomerular function, and no increase excretion of urinary cadmium was observed by clearance method.

Kobayashi et al. (2009)[107] performed ten year follow up renal handling in Ca exposed inhabitant after soil replacement in paddy field was done. They showed that the degree of renal tubular injury was not found to improve in either sex and the renal handling of Ca showed no or only a slight change through out the observation period in both sexes.

Schutte et al. (2008)[38] sought evidence for a direct osteotoxic effect of cadmium in women, concerning to hypercalciuria. Belgians environmentally exposed to cadmium were enrolled to this study. They showed association between urinary cadmium and increase urinary Ca excretion. The conclusion of this study was that the environmental exposure to cadmium increased bone resorption in women, suggesting a direct osteotoxic effect with increased calciuria.

Staessen et al. (1991)[15] determined Ca and bone metabolism in environmental cadmium exposure Belgian inhabitant. They showed that serum alkaline phosphatase activity significantly correlated to urinary Ca and positively correlated with urinary cadmium in both men and women. Serum total Ca concentration negatively correlated with urinary cadmium excretion in men. When urinary cadmium level increased twofold a urinary Ca level rose by 0.25 mmol/24 hr. They concluded that even at environmental exposure levels Ca metabolism was gradually affected, as cadmium accumulated in the body.

Wu et al. (2001)[17] determined the feasibility of urinary Ca as a marker of renal dysfunction induced by cadmium. Chinese exposed inhabitants were enrolled in this study. They showed a significant dose-response relationship between the

prevalence of hypercalciuria and the excretion of urinary cadmium. A significant increase prevalence of calciuria was shown when excretion of urinary cadmium exceeded 2 μg/g Cr. They concluded that cadmium exposure could result in increased excretion of urinary Ca in a general population. Urinary Ca can be used as a biomarker of renal dysfunction induced by cadmium.

1.10 Cadmium and anemia

One finding shown in itai-itai patient is severe anemia which was closely associated with an impaired renal function[5]. To clarify this relation a studies in human and animal were conducted.

Hiratsuka et al. (1996)[108] investigated the chronic cadmium exposure-induced nephrotoxicity and anemia in ovariectomized rats. Rats were treated with $CdCl_2$ for 50 weeks and determined their hematological parameters. Anemia occurred at 12 weeks in the 0.5 mg/kg treated group and became increasingly marked with time. Decrease in plasma iron levels and increase iron binding capacity were observed. Erythropoietin level was not elevated even the hemoglobin level was decreased at 50 weeks.

Horiguchi et al. (1994)[6] determined a relation between cadmium exposure and anemia among itai-itai patients. They found that low serum iron or ferritin levels were not observed, and bone marrow aspiration did not reveal any specific hematological disorders. A close relationship between the decrease in the hemoglobin level and the progression of renal dysfunction was observed. Low serum erythropoietin levels were detected despite the presence of severe anemia.

Horiguchi et al. (1996)[109] used rat model to prove that the anemia observed in chronic cadmium intoxication arises from low production of erythropoietin in the kidneys following the renal injury. After 6 and 9 month of cadmium administration, rats showed anemia with low levels of plasma erythropoietin as well as biochemical and histological renal tubular damage, and also hypoproduction of erythropoietin. They concluded that chronic cadmium intoxication caused anemia by disturbing the erythropoietin production capacity of renal cells.

Horiguchi et al. (2010)[110] also reported that cadmium polluted area inhabitant, who showed urinary β_2-MG > 3,000 μg/g Cr, had low-normal serum erythropoietin even decreased hemoglobin.

Moriguchi et al. (2003)[45] investigated relation between iron-deficiency and renal dysfunction among non-cadmium-polluted area inhabitants whose urinary cadmium mean was 1.41 μg/g Cr. They showed that after classified subjects into anemic and non-anemic groups, urinary cadmium and proximal tubular markers levels were not significantly different between these groups. The conclusion from this study was iron deficiency anemia showed no relation to proximal tubular dysfunction or urinary cadmium.

Satarug et al. (2004)[82] determined influence of cigarette smoking, body iron store status and gender on cadmium body burden in non-exposed Thais. They showed that women're cadmium body burden was inversely correlated with serum ferritin and those with low iron stores showed a 3.4-fold greater cadmium body burden than did women whose serum ferritin being between 101 and 200g/l. In contrast, men're cadmium body burden did not show a significant correlation with serum ferritin, but it did show a positive correlation with cumulative cigarette smoking index. They

concluded that iron status and cigarette smoking were found to be determinants of cadmium body burden in young adult Thai women and men.

Tsukarhara et al. (2003)[46] determined relation between iron-deficiency and cadmium body burden among women in general population in Japan. They showed that anemia subjects showed lower serum ferritin than healthy non-anemia subjects. No significant differences in urinary cadmium and low molecular weight protein level were observed between anemia and non-anemia subjects. They concluded that iron deficiency among women in the general population in Japan may not induce significant increase in cadmium body burden or cadmium-induced tubular dysfunction.

Anemia prevalence was also determined in Thai cadmium exposed inhabitants. Limpatanachote et al. (2009)[22] reported increased anemia prevalence as urinary cadmium level increased. Prevalence of anemia in urinary cadmium >20 μg/g Cr group was 56.8%.

Increase anemia prevalence was also found in renal insufficient patient. Cumming et al. (2004)[111] determined increase anemia prevalence in glomerular insufficient patient by using creatinine clearance method. They showed a strong association between reduced renal function and anemia. Estimated creatinine clearance <50 mL/min was associated with a three-fold increased risk of anemia in women and a five-fold increased risk in men.

McClellan et al. (2004)[43] determined prevalence of anemia in patients with chronic kidney disease. They showed that after controlling for other patient characteristics, a decrease glomerular function associated with increased prevalence of anemia. Odds ratio for hemoglobin $\leq$ 10 g/dL was 0.54 (0.49-0.60) and for

hemoglobin $\leq$ 12 g/dL was 0.68 (0.65-0.72) with each 10-mL/min/1.73 m^2 increase in glomerular filtration rate. Predictors of anemia were diabetes, female sex and race/ethnicity. They concluded that anemia increased as kidney function decreased.

1.11 Cadmium and biomarkers

Cadmium exposure markers

Kidney is an accumulation organ of cadmium and cadmium level in the kidney showed close relation to urinary cadmium[21]. Urinary cadmium was proposed as a suitable cadmium body burden indicator among chronically cadmium exposed inhabitant[112] whereas blood cadmium was used to indicate cadmium body burden of recent cadmium exposure[2, 85].

Cadmium had very long whole-body biological half-time. Järup et al. (1983)[2] determined biological half-time of cadmium in the blood of workers after cessation of exposure and found that the estimated half-time of blood cadmium ranged between 7.4-16.0 years. Järup et al. (1997)[85] also evaluated blood cadmium in Swedish workers and found that cadmium-induced tubular dysfunction was irreversible. Järup and Åkesson et al. (2009)[1] reported that urinary cadmium concentration was mainly influenced by the body burden of cadmium and was proportional to the concentration in the kidney. Cadmium was efficiently retained mainly in the kidney with a biological half-time of around 10-30 years. Assuming a linear relationship, urinary cadmium of 5 μg/g Cr (~5 nmol/mmol Cr) approximately corresponded to about 100 mg/kg cadmium in the renal cortex. In an autopsy study of the subjects without occupational exposure to cadmium, a urinary cadmium level of 1.7 μg/g Cr was

equivalent to a renal cadmium level of 50 μg/g. Blood cadmium was considered as the most valid marker of recent exposure.

Kido et al. (1992)[113] found that mean urinary cadmium concentration of Kakehashi River basin inhabitants who heavily exposed to cadmium increased in a dose-related manner when the subjects were classified according to the average cadmium concentration in rice grown in their village and the period of residence in the polluted area. Kido et al. (2004)[112] also reported a relation between individual cadmium concentration in urine and total cadmium intake.

Nishijo et al. (1995)[64] summarized that blood cadmium concentration could be used as an indicator of recent cadmium exposure and it showed strong correlation to urinary cadmium which was mainly in equilibrium with body burden. However, the relationship between cadmium concentration in blood and external dose such as cadmium concentration in consumed rice was scarce. Nishijo et al. (2004)[114] also summarized that more cadmium accumulated in tissues of women than that of men and age was the host factor showing a remarkable correlation with blood and urinary markers of cadmium exposure.

Sartor et al. (1992)[14] also found that urinary cadmium level in women was greater than men and residing place affected cadmium body burden. Their finding showed that smoking 20 cigarettes per day increased the cadmium body burden by about 63% at the age of 50. A significant increase in cadmium body burden was found in subjects who lived in the polluted areas and comsumed locally grown vegetables and well water.

Satarug et al. (2003)[21] summarized that human-tissue cadmium contents showed large variations among individuals, but sources of the variation remain

unknown. The exposure levels of 30-50 μg cadmium per day had been estimated for adults and these levels had been linked to an increased risk of bone fracture, cancer, kidney dysfunction and hypertension.

Renal dysfunction markers

Both glomerular and proximal tubular dysfunctions can be indicated by urinary low molecular weight protein and proximal tubular bound enzyme[3]; β_2-microglobulin (β_2-MG), N-acetyl-β-D-glucosaminidase (NAG), Kidney injury molecule I (KIM-I) and serum cystatin C. β_2-MG is a low molecular weight protein which is normally reabsorped back to blood circulation by proximal tubular cell and it will be excreted to urine when proximal tubular dysfunction appeared[115]. It was a sensitive marker to proximal tubular dysfunction[116] and urinary β_2-MG at the level >1,000 μg/g Cr was used to indicate an irreversible proximal tubular dysfunction [30].

NAG is a lyzosomal enzyme which is abundant in cells of the proximal kidney tubule[117]. It will be released into urine after proximal tubular cell injury. NAG showed high sensitivity to indicate early proximal tubular dysfunction in epidemiological study[118].

Kidney injury molecule I (KIM-I) is a novel biomarker to indicate renal dysfunction[119]. This molecule will be excreted into urine when proximal tubular dysfunction appear and it shows high stability in urine sample[120]. An increase of KIM-I level in urine was found in the patients with acute kidney injury[121]. It was proposed and investigated as an early marker to indicate renal dysfunction in Mae Sot people[122].

Glomerular dysfunction can be reflected by decreased clearance of inulin or creatinine[123]. However, these methods require skillful technical worker over a period of several hours and high costs[124]. Recently, serum cystatin C was recognized as a useful marker to indicate glomerular dysfunction and it showed higher accuracy and precision over the estimated glomerular filtration rate(GFR) [123, 124]. Cystatin C is a non-glycosylated basic protein with molecular weight of 13.2 kDa. It is produced at a constant rate in nearly every nucleated cells in human body[125]. It is freely filtered through a normal glomerular membrane, and is then reabsorbed almost entirely in the proximal tubules. Hence, the cystatin C concentration in human blood is closely related to the GFR[126]. Cystatin C concentration has not been shown to be significantly influenced by other factors such as muscular mass, inflammatory diseases, sex, age or diet[127]. Serum cystatin C at the concentration of 0.90 mg/l could be indicated GFR by $\geq$90 ml/min/1.73 m^2 with a sensitivity of 75% and a specificity of 92%[123]. Serum cystatin C level was not affected by inflammatory processes, age, sex, and muscle mass[128, 129].

Bone markers

Bone is a metabolically active tissue that undergoes continual remodelling, with resorption coupled to formation to control blood Ca levels[130]. Formation mechanism is a function of osteoblast to produce and mineralized bone matrix. A predominant product of osteoblast is type I collagen, which comprises 95% of the extracellular non-mineral bone matrix. Osteopontin, osteonectic and osteocalcin are also secreted by osteoblast to form osteoid in which mineralization occured. A functional enzyme to mineralize bone matrix is alkaline phosphatase which is

synthesized by osteoblast to mineralize osteoid. On the other hand, bone resorption is a function of osteoclast which is formed by osteoblast signaling. Osteoclasts attach to the bone surface and secrete acid and hydrolytic enzymes to resorb bone, releasing bone mineral and collagen fragments[131].

In order to indicate bone remodelling, there are markers to specify both bone formation and resorption. Bone formation markers are product of active osteoblasts expressed during different phases of osteoblast development. Osteocalcin is considered as a specific marker of osteoblast function. During bone formation, a small osteocalcin fraction is released to blood circulation by osteoblast and serum osteocalcin can be detected by immunoassay[52]. Osteocalcin was used to determine bone formation in hormone replacement therapy, osteoporosis and cancer patients. It showed advantage to indicate early bone formation after treatment[53, 132, 133].

Bone resorption markers are degraded product of bone specific type I collagen. N-telopeptide (NTx) and C-telopeptide (CTx) of type I collagen, deoxypyridinoline (DPD), bone sialoprotein (BSP) and tartrate-resistant acid phosphatase are released to blood circulation by type I collagen degradation. NTx, CTx and DPD are filtrated pass glomerulus and excreted to urine. Enzyme link immunoassay (ELISA) can be used to detect these bone resorption markers in urine with high sensitivity and specificity[52, 131, 134]. Even it was proposed as a bone resorption marker but high level of bone sialoprotein was found in mineralized tissue, therefore, it should be used in caution to reflect bone resorption[52]. Tartrate-resistant acid phosphatase measurement requires serum sample and its activity was rapidly degraded in a long time kept sample[52]. Moreover, NTx and DPD were used to

indicate bone fracture risk among elderly and they showed high stability in frozen urine sample[53, 134, 135].

1.12 Hypothesis

Mae Sot inhabitants have high risk to develop bone metabolic dysfunction and anemia even though they appear to have no clinical signs and symptoms of cadmium intoxication. The bone metabolic dysfunction could be developed by calcium reabsorption impairment which could appear when proximal tubular dysfunction occurs. A study to investigate bone metabolism and anemia development has not been conducted among Thai exposed cadmium inhabitants. Therefore, the hypothesis of this study was chronic environmental cadmium exposure could cause imbalance in calcium dynamics and accelerate bone resorption and anemia could be developed in accordant to renal tubular dysfunction. In addition, expression of osteocalcin, bone alkaline phosphatase, type I collagen, RANKL and OPG genes would be overexpressed after exposing to cadmium and might be one of the etiology of bone pathology induced by cadmium.

1.13 Study Objectives

1. To investigate whether Mae Sot inhabitants who exposed to environmental cadmium have an increased risk of osteopathy.
2. To determine anemia prevalence and identify anemia risk factors in the population.
3. To investigate the direct effect of cadmium on human fetal osteoblast like cell (hFOB) on the expression of osteocalcin, bone alkaline phosphatase, type I collagen, RANKL and OPG genes.