X-Ray Microanalysis at High Accelerating Voltage Detecting Aluminum Accumulation in Mouse Kidney after Short-Term Aluminum Administration

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Elemental analysis on Al was carried out by energy dispersive X-ray (EDX) microanalysis using high accelerating voltage at 300 kV with high permeability in thick samples at 1 μ m thickness obtained from 27 ddY mice administered with Al for 1 to 17 weeks. The quantities of Al detected in the lysosomes of the convoluted tubule cells of the kidney did not relate to the Al administration period. A significant difference in the quantity of Al between the administration group and the non-administration group was found by stochastic analysis with two-way factorial analysis of variance followed

by Fisher's PLSD for multi-comparison. However, the quantities of Al detected in the nuclei of the proximal convoluted tubule cells of the kidney suggested a correlation with the Al administration period. EDX microanalysis using thick sections and high accelerating voltage was useful in detecting the Al accumulated in the lysosomes and nuclei of the kidney cells. The EDX microanalysis was able to detect Al accumulated in the lysosomes and clarify the transitional changes of the quantities of Al detected during a short-term excessive Al administration.

Key words: aluminum, EDX microanalysis, high voltage electron microscopy, lysosome, mouse kidney

I. Introduction

Aluminum is one of the common elements often found in our daily lives. Many papers report that Al causes pathological and toxicological influences upon the living body [29, 35, 36]. Aluminum is also suspected to cause such diseases as dialysis encephalopathy, which is due to dialysis treatment of patients suffering from renal failure [7]. The treatments for renal deficiency in patients are hemodialysis and anti-inflammatory or antacid drugs. It is well known that hemodialysis liquids and anti-inflammatory or antacid drugs contain Al gel. After the use of such hemodialysis liquids and drugs were stopped, complications from dialysis encephalopathy decreased. As a result, Al was regarded as the etiology of dialysis encephalopathy [7, 17, 29]. In recent years, it is of concern that acid rain dissolves Al from the earth's surface into bodies of water due to changes in the earth's environment. The influences of Al manifest not only

in renal failures on dialysis but also in fish and plants [6]. As a result, Al dissolved in acidic water is estimated to be absorbed in the living bodies of animals and plants. It is important to clarify the influence of excess Al accumulation taken into the living body through drinking water. Heavy metals such as cadmium (Cd) and copper (Cu) are well known to accumulate in cells and cause morbid influences in the living body [13, 30, 37]. The detection of such poisonous metals in cells and tissues has not only been carried out with biochemical methods [27], but also with histochemical and cytochemical techniques [13, 32, 33]. However, the histochemical studies on Al taken into the living body and its accumulation in cells are still insufficient. Metal accumulation, such as of Cu and Cd, in the living body is supposed to cause such toxic symptoms as kidney failure [16, 30, 35]. The problem of Al accumulation at the present time is to clarify the influence of Al absorption with small quantities for long durations in living bodies. Usually, the detection of elemental accumulation in a cell is measured by examining via biochemical methods, the proteins and enzymes which are bound to the elements in serum. The specific protein caused by metal accumulation increased in the tissues,

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blood, and urine [15, 18]. It was reported that the metallothionine protein was related to the accumulation of Cu in the cytoplasm of the brain, liver, duodenum, and kidney when cell degeneration occurred from metal accumulation [14, 19].

Elemental analysis study by energy dispersive X-ray (EDX) elemental microanalysis allowed observation of ultrastructural changes at the electron microscopic level and simultaneous qualification and quantification of an element *in situ*. Using biological specimens, EDX elemental analysis was carried out with ultrathin sections at 0.1 μm thickness and conventional electron microscopy with accelerating voltages of 80–100 kV [37]. The sections submitted to EDX elemental analysis should be thin because the electron beam of conventional electron microscopy can transmit only thin sections. However, it is thought that using thicker sections is more advantageous because they contain more elements [20, 21]. EDX microanalysis using high accelerating voltage at 300 kV and thicker sections of 1.0 μm thickness demonstrated that Al conjugated with proteins like metallothionine, concentrated in lysosomes of digestive organs such as the duodenum, liver, and kidney [12]. In the study, the administered Al was detected in lysosomes with a high electron density in the macrophages of the duodenal mucosa, the hepatocytes of the liver, and the proximal convoluted tubule cells of the kidney.

In order to elucidate the relation between the quantitative changes of absorbed Al in the kidneys and postnatal development and aging of experimental animals, we attempted to study the elemental analysis of Al using high accelerating voltage at 300 kV with EDX microanalyzer, utilizing its high permeability in thick sections according to the previous experiment, and to observe the morphological changes in the lysosomes of convoluted tubule cells in the kidneys after excess oral administration with Al-chloride to mice for short-term periods from 1 to 17 weeks during the aging process.

II. Materials and Methods

Animals

Nine groups of ddY strain male mice, each consisting of 3 littermates, from postnatal week 1 to 3, 5, 7, 9, 11, 13, 15, and 17, (total 27 individuals) were used. The embryonic ages of mice were based on observation of the vaginal plugs of the female mice (vaginal plug=day 0) after mating. The pregnant mice were housed under conventional conditions, fed with normal mice chow (Clea CE2, Tokyo, Japan) with access to deionized water *ad libitum* (Japan SLC, Inc., Hamamatsu, Japan) until they were purchased and transferred to our laboratory on embryonic day 14. Then, they gave birth on embryonic day 18, when the postnatal ages of the newborn baby mice were counted as postnatal day 0. Thus, postnatal day 1 to week 17 mice, each consisting of 3 littermates, (total 27) were administered orally with deionized water containing 2% AlCl_3 (aluminum chloride, Nakarai Chemical, Kyoto, Japan), at pH 2.5, *ad libitum*

immediately after birth until 17 weeks. In case of newborn mice at postnatal day 1 to 14, the animals were administered with deionized water containing 2% AlCl_3 by means of mouth tubing once a day and fed with mother's milk *ad libitum*. After delivery the mothers were also fed with deionized water containing 2% AlCl_3 *ad libitum* and allowed to nurse the baby mice *ad libitum* until postnatal week 3. After each administration period of 1, 3, 5, 7, 9, 11, 13, 15, and 17 weeks, two mice were sacrificed under ether anesthesia and the kidneys were removed, cut into several small pieces, and fixed. As the negative control without administration, nine ddY strain mice were administered orally with deionized water for the same administration period (from 1 to 17 weeks), sacrificed under ether anesthesia and the kidneys were removed, cut into several small pieces, and fixed at respective periods. All the procedures used in this study relating to animals were in accordance with the protocol reviewed and approved by the guidelines of the Animal Research Committee of Shinshu University School of Medicine as well as the principles of laboratory animal care (NIH Publication No. 85–23, revised 1985).

Morphological observation

For light and electron microscopic morphological observation, tissues were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4, at 4°C, for 2 hr and post-fixed in 2% osmium tetroxide in the same buffer at 4°C, for 1 hr. Then, the tissues were dehydrated in a graded series of ethanol, substituted with propylene oxide, and embedded in epoxy resin (Epok 812, Oken, Tokyo, Japan). For light microscopic observation, thick sections were cut at 3 μm thickness on an OmU4 ultramicrotome (Reichert-Jung, Vienna, Austria), stained with 1% toluidine blue (Merck, Frankfurt, Germany) in phosphate buffer at pH 7.4 and observed with a light microscope (Nikon Optiphot-2, Nikon, Tokyo, Japan). For electron microscopic observation, ultrathin sections were cut on the same ultramicrotome (Reichert-Jung, Vienna, Austria) at 0.1 μm thickness, mounted on copper grids (VECO, Eerbeek, Netherlands), and stained with uranyl acetate and lead citrate. Specimens were then observed with a conventional TEM, JEM-1200EX (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

EDX microanalysis

For EDX elemental microanalysis with electron microscopy, tissues were fixed only in 2.5% glutaraldehyde in phosphate buffer, pH 7.4, at 4°C, for 2 hr, dehydrated in a graded series of ethanol, substituted quickly with propylene oxide, and embedded in Technovit VL7200 resin (Meiwa, Tokyo, Japan). This Technovit VL7200 resin did not include chlorine and was suitable for elemental analysis. For elemental analysis of Al, thick sections were cut at 1.0 μm thicknesses on the same ultramicrotome, mounted on nylon grids (VECO, Eerbeek, Netherlands), stained with uranyl acetate, and coated with a thin carbon layer. The sections were then observed and analyzed in a high voltage TEM, JEM-4000EX (JEOL, Tokyo, Japan) at an accelerating

Fig. 1. Light micrographs of the kidneys of mice either administered without (A) or with Al for certain periods (B–F), fixed in glutaraldehyde and osmium tetroxide, embedded in Epok, sectioned at 3 μ m, and stained in TB. (A) The kidney tissue of a mouse administered with Al for 1 week. There were two types of tissues observed in the convoluted tubule cells of the kidney. One had many darkly stained granules (*), while the other was stained brightly (arrows). (B) The kidney tissue of a mouse administered with Al for 5 weeks. The number of darkly stained granules decreased as compared to that of mice administered Al for 1 week. (C) The kidney tissue of a mouse administered with Al for 9 weeks. The number of dark stained granules increased again. (D) The kidney tissue of a mouse administered with Al for 13 weeks. Many darkly stained granules and apical vesicles were seen in the same cells of the convoluted tubule. In addition, the degenerative changes of swelling cytoplasm in the proximal convoluted tubule cell were seen (*). (E) The kidney tissue of a mouse administered with Al for 17 weeks. Many darkly stained granules and apical vesicles were seen in the same cells of the convoluted tubule (arrows). Cell degeneration was seen with the disappearance of the nucleus in the convoluted tubule cells (*). (F) The kidney tissue of a normal control mouse fed on diet without Al administration for 17 weeks. Darkly stained and large granules were not observed in the proximal convoluted tubule cell. However, a few apical vesicles were seen in the proximal convoluted tubule cell. $\times 200$.

voltage of 300 kV, equipped with a TN-5400 EDX micro-analyzer (Tracor-Northern, Wisconsin, USA). The section thickness at 1.0 μm and the accelerating voltage at 300 kV were decided according to the data obtained from the relationship between accelerating voltages and P/B ratios of Al contained in the macrophages of mouse small intestine in sections of various thicknesses, from 0.1 to 2.0 μm , as was reported previously [12]. Electron beams (beam current 93 μA) were focused onto the highly electron-dense lysosome and nucleus in each proximal convoluted tubule cell of the kidney. Electron beams were continuously irradiated to a small spot (diameter 200 nm) and the emitted X-ray was analyzed with specific EDX spectra counts for 100 sec within 25% dead time. The Al spectrum was counted at the peak of Al K- α at 1.49 KeV energy and a sub-area was calculated at ± 0.1 KeV. The quantification of Al was measured by calculating a peak (P) to background (B) ratio. Background was counted within a range of 4.6–4.8 KeV, which was not measured at the specific peak in a biological specimen. The relative concentration of Al at each administration period was represented by the mean P/B ratio obtained from 10–15 randomly selected lysosomes and nuclei. The data were calculated as means $\pm$ standard deviations. Significant differences between the Al administration and the duration of administration were stochastically analyzed with repeated measurement using two-way factorial analysis of variance followed by Fisher's PLSD for multi-comparison.

III. Results

Light microscopic observation

Upon observing the kidney tissues of mice that orally administered with AlCl_3 by light microscopy, many darkly stained granules were found in the proximal convoluted tubule cells stained with toluidine blue (TB stain). The results of these observations at each administration period from 1 to 5, 9, 13, and 17 weeks are shown in Figure 1. At 1 week of Al administration, two types of structures were observed: one in which the convoluted tubule cells contained dark granules and the other in which the convoluted tubule structures were seen brightly in the kidneys of the Al administered mice (Fig. 1A). In the portions where the convoluted tubule structures were formed, the proximal convoluted tubule cells and the distal convoluted tubule cells were clearly distinguished by light microscopy. However, those structures showed no difference from the normal

control mice 1 week after birth without Al administration. In the early newborn period, the proximal convoluted tubule cells of the Al administered mice contained many darkly stained granules, which were supposed to be the active re-absorption in the kidney. At 5 weeks Al administration, the numbers of black granules in the proximal convoluted tubule cells had decreased and the intensity of TB staining weakened compared to the period after 1 week administration (Fig. 1B) as well as with normal control animals at postnatal 5 weeks. At 9 weeks Al administration, the number and intensity of the black granules in the proximal convoluted tubule cells increased again (Fig. 1C). At the same time, small vesicles in those cells increased in number around the urinary lumen (Fig. 1C). Such changes were not found in the normal control animals at postnatal 9 weeks. At 13 weeks Al administration, the interstitial regions in the tubule cells disappeared as a result of proximal convoluted tubule cells that degenerated with swelling changes (Fig. 1D). In addition, nuclei of the proximal convoluted tubule cells partially disappeared and the number of the granules increased to a level greater than that of the previous period at 9 week administration. The brush borders of the proximal convoluted tubule cells were clearly observed in the apical cytoplasm, though they appeared brightly because of their cytoplasmic degeneration. Such changes were not found in the normal control animals at postnatal 13 weeks. At 17 weeks, many black granules and apical vesicles were observed in proximal convoluted tubule cells (Fig. 1E). The interstitial tissues in the tubule cells disappeared because of degeneration. The degeneration of tissue structures was also observed in the kidney tissues at 17 weeks administration (Fig. 1E). Those changes were not found in the normal control animals without Al administration at postnatal 17 weeks (Fig. 1F).

Electron microscopic observation

The results obtained from ultrastructural observation on proximal convoluted tubule cells of mouse kidney tissues at 1 week after Al administration as well as the negative control without Al administration are shown in Figure 2. In the negative control animals that were not administered with Al, the lysosomes with high electron densities were fewer in number and smaller in size (Fig. 2A) than the experimental animals that were administered with Al (Fig. 2B). Apical vesicles were seen in the portion near to the urinary lumen in the proximal convoluted tubule cells of both the administered and un-administered animals. At 1 week after Al administration, many large sized lysosomes with high electron

Fig. 2. Electron micrographs of the proximal convoluted tubule cells of mice, either administered without (A) or with Al for 1 week (B), 5 weeks (C), 9 weeks (D), 13 weeks (E), and 17 weeks (F), fixed in glutaraldehyde and osmium tetroxide, embedded in Epok, sectioned at 0.1 μm , and stained with uranyl acetate and lead citrate. (A) A very small lysosome (arrow) and small vacuoles (arrow head) were seen near the urinary lumen. (B) Large lysosomes (arrow) and vacuoles (arrow head) were seen around the nuclei. (C) Large vacuoles (arrow head) were seen around the urinary lumen. (D) Many lysosomes (arrow) and small vacuoles (arrow head) were seen near the urinary lumen. (E) The number of large (*) and small (arrows) lysosomes increased in the proximal convoluted tubule cells. Cellular degeneration (**) was also seen in the proximal convoluted tubule cells. (F) Large (*) and small (arrows) lysosomes were seen in the proximal convoluted tubule cells. The large lysosomes were seen in the sites where the nuclei are usually located in the proximal convoluted tubule cells. Bars=2 μm .

Fig. 2

densities were observed (Fig. 2B). In addition, there were large vacuoles in the proximal convoluted tubule cells in the cytoplasm near to the urinary lumen, while a small vacuole was observed in the portion surrounding the nucleus. The results obtained from the ultrastructural observation of the proximal convoluted tubule cells of mice kidneys at each AI administration period from newborn to 5, 9, 13, and 17 weeks are shown in Figures 2C, D, E, and F, respectively. At 5 weeks AI administration, the number of the lysosomes with high electron densities decreased (Fig. 2C) as compared to that of 1 week AI administration (Fig. 2B). The size of the lysosomes administered with AI for 5 weeks was observed to be smaller than the size of lysosomes administered for 1 week. Nevertheless, the numbers of various-sized vesicles at 5 weeks AI administration were observed more than those at 1 week administration period. Observations by high power magnification revealed that the structures of some lysosomes contained heterogeneous bodies with high electron densities, which should be classified as heterolysosomes in the proximal convoluted tubule cells at 5 weeks administration. At 9 weeks AI administration, the number of lysosomes with high electron densities as well as the number of degeneration vacuoles increased (Fig. 2D). At 13 weeks AI administration, the lysosomes with electron density and

degeneration vacuoles increased to levels more than those in 9 week administration (Fig. 2E). Under high magnification, evidence of degeneration and swelling of the cytoplasm in some proximal convoluted tubule cells was seen to be similar to that of the 9 week administration group. In addition, it was recognized that the brush borders of proximal convoluted tubule cells disappeared in the degenerative cells. Those results suggested that such cell degeneration caused illness with proteinurea, amino acid-urea, and glycosuria in the kidney. At 17 weeks AI administration, the lysosomes with electron density and degeneration vacuoles were also observed in the proximal convoluted tubule cells (Fig. 2F). The size of electron-dense lysosomes at this period was the largest among the 9 groups.

EDX microanalysis

The AI measurement by EDX microanalysis was carried out in the lysosomes with high electron densities in the proximal convoluted tubule cells of the mouse kidney, which were administered with AI from newborn to 17 weeks after birth. The electron micrograph and the spectrum of EDX microanalysis obtained from the lysosomes and the nuclei in the proximal convoluted tubule cells of the mouse kidney that were administered with AI for 9 weeks, are

Fig. 3. An electron micrograph and two EDX spectra in a proximal convoluted tubule cell of the kidney with AI administration for 9 weeks. (A) The lysosome (arrow) and the nucleus (arrow head) were shown in the proximal convoluted tubule cell of the kidney, fixed in glutaraldehyde, embedded in Technovit resin, sectioned at 1 μ m, and stained with uranyl acetate only. L, Lysosome; N, Nucleus; BM, Basement membrane; BB, Brush border. Bar=1 μ m. (B) EDX spectrum obtained from a highly electron dense lysosome (arrow in A) showing Al peak (arrow). (C) EDX spectrum obtained from the nucleus (arrow head in A) showing Al peak (arrow).

Fig. 4. Transitional changes of Al P/B ratios obtained from the lysosomes and the nuclei of proximal convoluted tubule cells of mice administered with or without Al for periods from 1 to 17 weeks. (A) Al P/B ratios in the lysosomes. The ratios of the Al administration group were higher than the non-Al administration group. The significant difference by two-way factorial analysis of variance followed by F-test was at $p < 0.0001$. (B) Al P/B ratios in the nuclei. The Al administration group were higher than the non-Al administration group. The significant difference by the two-way factorial analysis of variance followed by F-test was at $p < 0.048$.

shown in Figure 3. In the specimens for EDX microanalysis, not enough contrast existed to observe ultrastructures by electron microscopy because we did not use either osmium tetroxide for post-fixation or lead citrate for electron staining (Fig. 3A). If we had used both osmium tetroxide and lead citrate in specimens preparation, artifacts would have been produced when elements were analyzed by EDX microanalysis. We were able to detect Al in this study by selecting and precisely localizing the lysosomes for analyzing Al, which had high electron density without the post-fixation and lead staining. Likewise, the nuclei of the proximal convoluted tubule cells were selected and analyzed.

The EDX spectra obtained from the lysosomes (Fig. 3B) and the nuclei (Fig. 3C) in the proximal convoluted tubule cells showed not only Al but also sodium (Na), silica (Si), phosphorus (P), sulfur (S), chlorine (Cl), uranium (U), potassium (K), calcium (Ca), iron (Fe), and copper (Cu). There was a lot of Na, Si, and Cu, in addition to Al in the lysosome (Fig. 3B), while there were large amounts of Si and P in the nucleus (Fig. 3C). The elements K and U were detected in a complex peak in the spectra by EDX microanalysis. Uranium was used with electron staining for ultrastructural observation. As for Fe and Cu in the spectra, they may be artifacts that were driven from the specimen chamber at the periphery of the electron microscope. The transitional changes of Al P/B ratio with administration periods obtained from the EDX microanalysis, in both the lysosomes and the nuclei that were administered with and without Al administration from newborn to 17 weeks, are shown in Figure 4. Here, the highest peak of Al P/B ratio is suggested to be at the period after Al administration for 1 week. Comparing the P/B ratios of Al in the lysosomes between the two groups of the experimental animals with Al and without Al, the former

were higher than the latter at respective administration periods (Fig. 4A). The stochastic analysis on the significant differences between the quantities of Al in the lysosomes of the kidneys of mice in the two experimental groups with and without Al administration, as well as the transition during the administration, were calculated using factorial design with repeated measure two-way factorial analysis of variance followed by Fisher's PLSD for multi-comparison. The results showed that the significance of Al administration in the lysosome was at $p < 0.0001$, and the significance of administration periods was at $p < 0.015$. Thus, both the differences were significant at $p < 0.015$. The results showed that the significance of Al administration in the nuclei was at $p < 0.001$, and the significance of administration periods was at $p < 0.048$. Thus, both the differences between the nuclei in the two groups were significant at $p < 0.05$.

IV. Discussion

In the present study, the morphological changes of the lysosomes in the kidneys of mice were observed in the experimental animals to which excess Al was administered. In addition, EDX microanalysis using thick sections observed by high accelerating voltage electron microscopy at 300 kV confirmed the Al accumulation in the lysosomes and the nuclei in the proximal convoluted tubule cells of the kidneys of Al administered mice. It is generally accepted that Al orally administered is absorbed by the gastrointestinal tract and accumulated in various organs of experimental animals [26–28]. When Al was orally administered to such experimental animals as mice and rats, the concentrations of Al in the plasma [18] and urine [15, 26–28] rose within 2 hr after administration. The authors also showed that

administered Al was accumulated in the duodenum, the liver, and the kidney within a short time by means of biochemical analyses or histochemical stainings [27]. Those data suggested that Al, which was orally administered and absorbed from gastrointestinal tracts, was carried to the various organs through blood circulation and finally excreted into the urine.

Concerning the pH of the drinking water given to the experimental animals, it is not ideal to use such a low pH solution as aqueous AlCl_3 at pH 2.5. However, the efficiency of the Al absorption with low pH aqueous solutions in the gastrointestinal tracts, as well as the accumulation into the blood and tissues, had already been reported, showing that it was much better than at a higher pH [26]. Our previous experiment confirmed that some growth failure was seen in the mice treated with AlCl_3 administration. Almost all mice were alive during the administration periods until postnatal 17 weeks without any problems.

The morphological changes in the kidney tissues of mice due to prenatal development and aging were already studied by means of light and electron microscopic radioautography using ^3H -thymidine, ^3H -uridine, and ^3H -glucosamine incorporations in our laboratory [8, 10]. It was demonstrated that the DNA synthesis, proliferation, and the developments of both renal corpuscles and the uriniferous tubules including proximal convoluted tubule cells, were completed by postnatal day 1, in contrast to the results that the RNA and glycoprotein syntheses decreased from prenatal stage to postnatal 1 week and still continued until postnatal year 1, which showed the turnovers of these molecules. Thus, the detection of Al in the proximal convoluted tubule cells from postnatal week 1 to 17 in this study was carried out after the morphogenesis was completed in mice.

Previously, many studies reported on applications of EDX microanalysis to biological samples [4, 9, 34]. In those studies, conventional electron microscopy and ultrathin sections at 0.06–0.1 μm thicknesses were used with various methods of the fixations. Usually, biological researchers use conventional electron microscopy with ultrathin sections at 0.1 μm by EDX microanalysis which is carried out at accelerating voltages of 80–100 kV. The reason for using ultrathin sections at 0.1 μm thicknesses is that electron beams at accelerating voltages of 80–100 kV can not transmit sections thicker than 0.1 μm ultrathin thickness. However, it is obvious that very small amount of any element can be detected more efficiently in thicker sections, which contain many more elements than do thinner sections. We have already reported several studies in which it was suggested that EDX microanalysis using high accelerating voltages and thicker sections could efficiently detect a very small amount of elements such as Al, Ag, Ce, Zn, Ca, and S [11, 12, 20–22, 24, 25]. The utility of using high acceleration voltages by electron microscopic observation of thick biological samples was formerly reported by the present authors [11, 12, 20–22].

On the other hand, in order to detect all the elements contained in the biological samples by EDX microanalysis,

some specific fixations, other than conventional chemical fixations such as glutaraldehyde and osmium tetroxide, should be applied. For detecting electrolyte and insoluble components, freeze fixation followed by freeze-drying or freeze-substitution and embedding in resin without using any water, is necessary [4, 9, 23]. The methods for freeze fixation followed by freeze-drying or freeze-substitution and embedding in resin or freeze-dried cryosections did better at keeping all the elements in the tissues and cells by preventing the washing-away and diffusion of the soluble components, compared with other conventional methods [23]. However, it was ascertained that few elements were preserved with chemical fixation on EDX microanalysis [11]. It was also supposed that some elements bound to the insoluble components were preserved in the biological samples fixed with chemical fixation. In the previous study, it was suggested that Al conjugated with proteins was preserved in the lysosomes and nuclei [12]. Thus, in the present study, we used chemical fixation for detecting Al by EDX microanalysis in the kidneys during the administration periods.

As a result of the morphological observations in the present study, changes in the lysosomes of the kidneys by excess Al administration were found to be similar to those caused by lysosomotropic metals such as Cu, Fe, Hg, Au, Tl, as reported by De Duve *et al.* [5] and Schneider *et al.* [31]. They reviewed the biochemical and morphological changes in the lysosomes caused by lysosomotropic agents, which included not only the metal agents but also various drugs such as antibiotics, phenothiazine derivatives, anti-inflammatory and anti-parasitic drugs. They discovered administered elements such as copper, iron, mercury, gold, thallium, and silica, in the lysosomes of the liver and other organs in humans and other animals. Concerning Al accumulation, it was shown that overload or depletion of iron affected the uptake of Al into the sera, urine, and brains of rats [2]. Likewise, human hemodialysis patients who were exposed to an accidental aluminum overload, showed dramatic increases in the proportion of plasma aluminum levels to plasma iron levels [3]. When traces of metals such as aluminum, gallium, indium, zirconium, hafnium, chromium, niobium, uranium, gadolinium, gold, nickel, palladium, and platinum were abnormally introduced into experimental animals *in vivo* or *in vitro*, it was demonstrated that those metals selectively accumulated in the lysosomes of the convoluted tubule cells of the kidney by EDX microanalysis [1]. Schneider *et al.* reviewed in detail that such changes of lysosomes were called, 'lysosomal storage disease' [31].

On the other hand, Menkes' disease was known to be a congenital abnormality of copper metabolism [13], while the macular mouse was well known to be a model animal for Menkes' disease. In the macular mouse kidney, similar changes in the lysosomes of the convoluted tubule cells were reported [33]. Thus, Menkes' disease is caused by a copper storage problem in the lysosomes of the kidney. In those studies, the specific lysosomes increased when they were injured by the accumulation of copper [13, 33]. In the present study on Al accumulation, the morphological changes of the

lysosomes in the proximal convoluted tubule cell of the kidney were observed to be similar to those of Menkes' disease [13]. Furthermore, the size and the number of lysosomes observed in the convoluted tubule cells in the kidney changed depending on the administration period when Al administration was maintained. The Al contents in the lysosomes and the nuclei in the proximal convoluted tubule cells were directly analyzed by EDX microanalysis in this study. As a result, an important conclusion provided in this study on an Al accumulation model in experimental animals was that Al accumulation was directly shown in the lysosomes and nuclei. Aluminum in lysosomes was detected in the proximal convoluted tubule cells of the kidney, and the quantities of Al increased according to the administration periods. The quantity of Al in the nuclei also increased according to the administration periods.

It was reported that Al administration inhibited the lysosomal proton pump in rat liver [38]. The authors confirmed that administered Al was either combined with proteins and accumulated in the lysosome or that it remained as an electrolyte ingredient in the cytoplasm. It was estimated that Al of an existing electrolyte ingredient, controlled activity of proton pump in the lysosome of cytoplasm. As the result, the possibility that Al may be obstructed from being taken into the lysosomes was interrupted by excessive Al incorporation in cytoplasm, which caused hinderance of the formation of Mg-ATP complex in the lysosome membrane. In the present study, the cell injuries of the convoluted tubule cells were observed to advance depending on the Al administration period; even the quantities of Al in lysosomes did not increase. As a result of ultrastructural observation, cytoplasm degeneration and the disappearance of brush borders in the proximal convoluted tubule cells of the kidney were seen at 13 weeks after Al administration. The appearances of large lysosomes, which were seen from 13 to 17 weeks administration with Al, were possibly due to the excess accumulation of Al in the nuclei, caused the nuclear degeneration, so that perhaps the Al components in the karyoplasm were taken into the lysosomes.

In conclusion, the present study dealt with EDX microanalysis using thick sections and high accelerating voltage electron microscopy detecting Al accumulation in the proximal convoluted tubule cells of the kidney of mice, which were administered with Al for short-term periods. The quantities of Al detected in the lysosomes of convoluted tubule cells of the kidney did not relate to the Al administration period. The significant differences in the quantities of Al in the lysosomes and nuclei were observed by repeated measurement using two-way factorial analysis of variance followed by Fisher's PLSD for multi-comparison, in which the administration group and the non-administration group were compared. Furthermore, the quantity of Al detected in the nuclei of the convoluted tubule cells of the kidneys of the Al-administered mice showed a correlation with Al administration period.

It was suggested that the EDX microanalysis with thick sections and high accelerating voltage was useful in

analyzing metal accumulations in biological samples.

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