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Intranuclear iron deposition in hepatocytes and renal tubular cells in mice treated with ferric nitrilotriacetate

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Abstract Cytoplasmic and intranuclear iron depositions were observed in the livers and kidneys of male and female ddY mice treated for 4–12 weeks with ferric nitrilotriacetate (Fe-NTA), a known renal carcinogen that acts through the production of free radicals. The intranuclear iron deposition consisted of a spherical aggregation of ferritin particles of approximately 10 nm diameter, as revealed by immunohistochemical staining and electron microscopy. Although the incidence of renal tumours was greater in the males than in the females, the incidence of iron depositions did not differ with gender. The most abundant intranuclear iron depositions were observed in the animals treated with Fe-NTA for the longest duration (12 weeks). These findings suggest that the intranuclear production and propagation of free radical reactions are prevented by the trapping of iron in a chemically inert iron form of ferritin.

Key words Intranuclear iron deposition · Ferric nitrilotriacetate · Ferritin · Renal tumours

Introduction

We have previously reported that the parenteral administration of iron-chelated nitrilotriacetate (Fe-NTA) causes haemochromatosis-like cytoplasmic iron deposition in the parenchymal cells of the rat liver, pancreas [1] and kidneys [24, 25]. The oral administration of carbonyl iron to rats also induces iron accumulation in the cytoplasm of duodenal mucosa and liver cells [2, 20, 22, 27]. In addition, the injection of Fe-NTA causes severe acute renal tubular necrosis and induces renal tumours in rats and mice through an iron-catalysed free radical reaction [24]. The incidence of renal tumours and the extent of renal tubule lipid peroxidation are greater in male than in female rats and mice [10, 18, 19, 26, 30]. Our previous data indicated that the gender difference observed in the incidence of renal tumours induced by Fe-NTA was dependent on sex hormones [10].

In contrast, intranuclear deposition of iron has rarely been observed after the administration of iron compounds. Descriptions of intranuclear iron depositions in the kidney are few, although intranuclear lead deposits in the kidney have been observed in the mute swan [5]. Haddow and Horning [15], Goetz and Richter [12] and Smith et al. [29] described intranuclear iron-positive inclusions in the hepatocytes of mice after the administration of iron dextran or saccharated iron oxide. Repeated injections of iron sorbitol and iron polymaltose to baboons resulted in intranuclear ferritin-like particles in some hepatocytes, endothelial cells and Kupffer cells [14]. Iron-containing particles in the nuclei of spleen cells were also observed in mice given blood transfusions [17] and in rabbits after injections with colloidal iron [21]. Cai et al. reported the presence of nuclear ferritin in avian corneal epithelial cells [8].

Here we describe the simultaneous depositions of iron in the cytoplasm and nuclei of liver and kidney cells of mice treated with Fe-NTA for 12 weeks. In addition, although the induction of tumours was seen only in the kidneys and the tumour incidence was higher in the male mice, the incidence of iron deposition was the same in the male and female animals, and the iron deposition was more abundant in the livers than in the kidneys.

Materials and methods

All reagents used were of the highest quality available. Nitrilotriacetic acid (NTA) disodium salt was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents (unless specified) were purchased from Wako Pure Chemicals (Osaka, Japan). The polyclonal antibody against human liver ferritin (AB999) was provided by Chemicon (Temecula, Calif.). Avidin-biotinylated peroxidase

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Table 1 Treated A groups, treatment and summary of iron deposition (*n* number of surviving animals in each group; *Cas* castration, *Est* estradiol treatment, *Ov* ovariectomy, *Test* testosterone treatment)

Group (*n*)	Sex	Treatment	Number (%) of animals positive for iron			
			Hepatocyte		Renal cell	
			Cytoplasm	Nucleus	Cytoplasm	Nucleus
A-1 (16)	M	Sham-FeNTA	14 (88%)	16 (100%)	4 (25%)	1 (6%)
A-2 (17)	M	Cas+FeNTA	13 (77%)	13 (77%)	5 (29%)	0 (0%)
A-3 (5)	M	Cas+FeNTA+Est	5 (100%)	1 (20%)	2 (40%)	0 (0%)
A-4 (18)	F	Sham+FeNTA	17 (94%)	16 (89%)	5 (28%)	0 (0%)
A-5 (17)	F	Ov+FeNTA	14 (88%)	15 (88%)	14 (82%)	5 (29%)
A-6 (7)	F	Ov+FeNTA+Test	7 (100%)	7 (100%)	7 (100%)	2 (29%)
A-7 (10)	M	Sham+NTA	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 2 Treated B groups, treatment and summary of iron deposition (*n* number of animals survived in each group)

[a]* Significantly different from groups 2–4 by the χ^2 test ($P<0.05$)
[b]* Singificantly different from groups 3–4 by the χ^2 test ($P<0.05$)

Group (*n*)	Injection	Duration of injection (weeks)	Number (%) of animals positive for iron			
			Hepatocyte		Renal cell	
			Cytoplasm	Nucleus	Cytoplasm	Nucleus
B-1 (12)	FeNTA	12	11 (92%)	6 (50%)[a]*	8 (67%)	4 (33%)[b]*
B-2 (11)	FeNTA	8	9 (82%)	0 (0%)	5 (45%)	1 (9.1%)
B-3 (17)	FeNTA	4	12 (71%)	2 (12%)	0 (0%)	0 (0%)
B-4 (10)	NTA	12	0 (0%)	0 /0%)	0 (0%)	0 (0%)

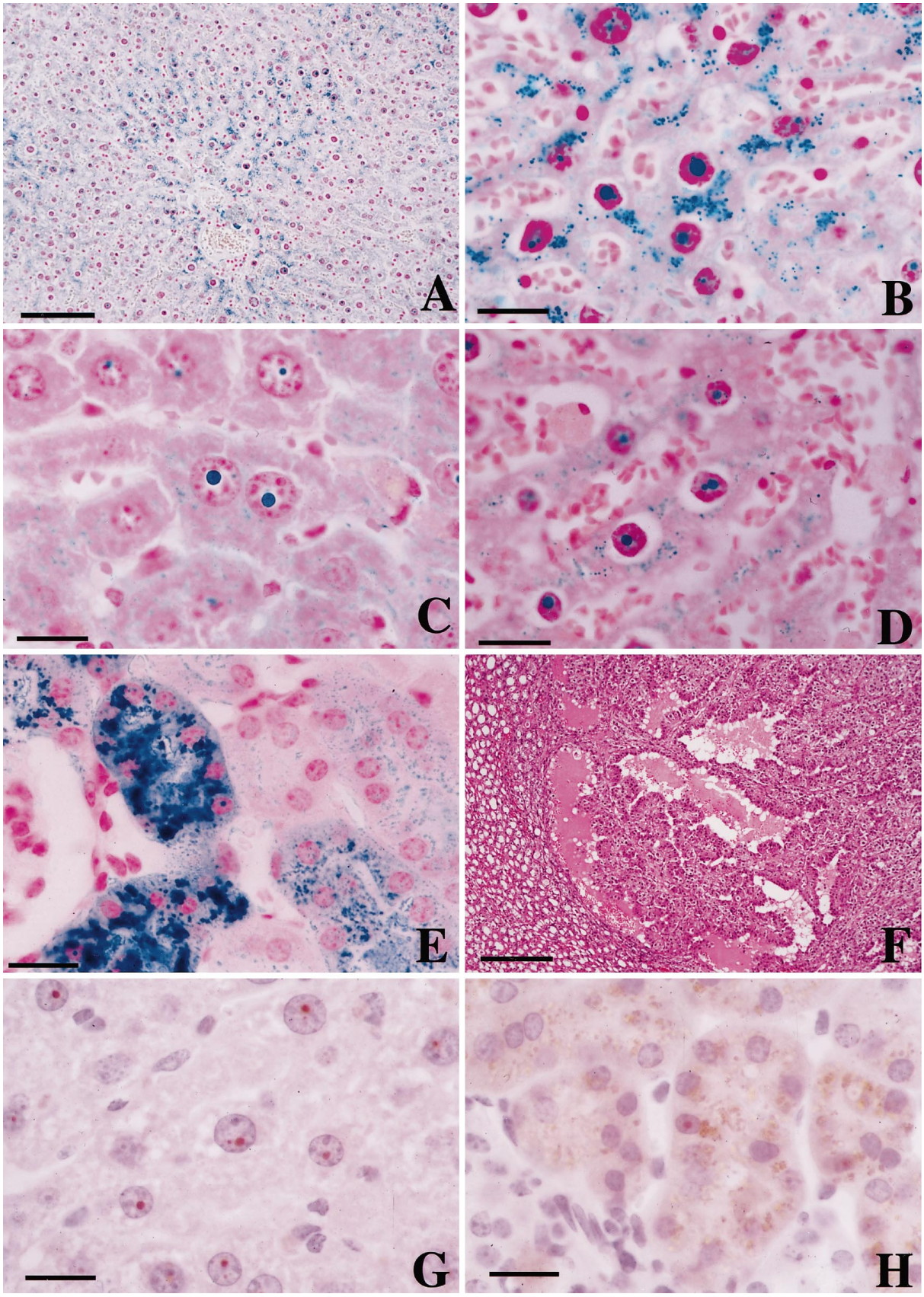
complex (ABC) was provided by Vector Laboratories (Burlingame, Calif.). Fe-NTA was prepared by the method of Awai et al. [1]. Briefly, NTA disodium salt and ferric nitrate were separately dissolved in distilled water, and the iron solution was mixed with the NTA solution. The pH was adjusted to 7.2 with sodium bicarbonate. The molar ratio of iron to NTA was 1:4, and the iron concentration was 1 mg/ml.

Four-week-old male and female ddY mice were purchased from Shizuoka Laboratory Animal Centre, Shizuoka, Japan and fed a commercial mouse chow (Funabashi Fl, Chiba, Japan) with tap water ad libitum. Male and female animals were randomly divided into seven groups within group A, and only male animals were divided into four groups within group B. Tables 1 and 2 show the experimental groups and treatment conditions; and Table 2 also shows the durations of injection. The animals in groups A-1, A-4 and A-7 were sham-operated, and the animals in groups A-2, A-3, A-5 and A-6 were castrated or ovariectomized under pentobarbital anaesthesia within 1 week after purchase, after which they were allowed to recover untreated for 4 weeks. The animals in group A-3 were each given an i.m. injection of 5 mg/kg estradiol valerate (Pelanin Depot, Mochida, Tokyo) twice a week for 10 months. Those in group A-6 received 10 mg/kg testosterone propionate (Testinon, Mochida, Tokyo) i.m. once a week for 10 months. Fe-NTA was administered i.p. from 9 weeks of age onward. The animals in groups A-1 to A-6 were given Fe-NTA at 2–8 mg of iron/kg per day i.p. six times a week for 12 weeks. We started with 2 mg of iron/kg per day and increased the dose up to 8 mg of iron/kg per day. Iron injection was suspended when there had been no weight gain since the previous injection. The animals in groups B-1, B-2 and B-3 were given Fe-NTA at 10 mg of iron/kg per day twice a week for different durations. For reference, several animals were given same treatment for 1 or 2 weeks. In groups A-7 and B-4 the NTA dose was equivalent to the NTA portion of Fe-NTA injected in the animals in the other groups. During the experiment, sick animals were sacrificed at 1 or several months after the treatment for reference. Twelve months after the first treatment, all animals in the A and B groups were sacrificed under ether anaesthesia. The numbers of animals that survived are shown in Tables 1 and 2. The livers and kidneys were horizontally sectioned, fixed in 10% phosphate-buffered formalin, and embedded in paraffin. They were sectioned at a thickness of 4 μm and stained with haematoxylin-eosin for routine examination.

One of the serial paraffin section from the liver and one from the kidney of each mouse was stained for iron according to Perls' iron staining method [28]. Perls' stain was also performed after the dewaxed thin section with Bouin's solution (pH 1.0 by acetic acid) has been rinsed for 120 h at 37° C, for a rough estimate of the hydrophilicity of the iron deposit. The other sections were used to determine the immunohistochemical localization of ferritin according to the avidin–biotin–peroxidase complex (ABC) method using a Vectastain ABC kit (Vector). The primary anti-human ferritin antibody, which was confirmed to cross-react with mouse liver ferritin [16], was diluted to 1:500 and reacted with tissue sections at 37° C for 30 min. The amino acid sequences of human ferritin subunits show an 82% homology, and antibodies to L-determinants of human ferritin recognize the major subunit of mouse liver ferritin [3, 4].

Paraffin blocks of the livers and kidneys of the mice were dewaxed and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4° C, postfixed in 1% buffered osmium tetroxide for 1 h at 4° C, dehydrated, and embedded in Epon 812 by the usual

Fig. 1 Localization of iron in Fe-NTA-treated mice by Perls' iron staining. **A** Stainable iron was more dominant in the peripheral area than in the central area of the liver lobule. Perls' iron staining, ×200, *bar* 100 μm. **B** Liver section in mice 1 year after a 12-week Fe-NTA injection. Large round iron depositions were observed in the nuclei with several large nucleoli beside them. Perls' iron staining, ×1000, *bar* 20 μm. **C** In another liver section, iron depositions were observed in the nuclei but no iron depositions were observed in the cytoplasm. Perls' iron staining, ×1000, *bar* 20 μm. **D** A section rinsed in Bouin's solution, pH 1.0, for 120 h. Intranuclear iron was resistant to the acid treatment, while cytoplasmic iron was decreased. Perls' iron staining, ×1000, *bar* 20 μm. **E** In the kidneys of mice treated with Fe-NTA for 12 weeks, small intranuclear iron depositions were observed in the renal tubular cells. Perls' iron staining, ×1000, *bar* 20 μm. **F** Cystic and papillary proliferation are seen in the kidney 12 months after the start of Fe-NTA treatment. HE, ×100, *bar* 200 μm. **G** Intranuclear and weak cytoplasmic iron depositions are positively stained for ferritin in the liver. ABC staining, ×1000, *bar* 20 μm. **H** In the renal proximal tubular cells, intranuclear and cytoplasmic iron depositions are also positively stained for ferritin. ABC staining, ×1000, *bar* 20 μm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method. Ultrathin sections were cut using an ultramicrotome (Ultracut E, Reichert, Heidelberg, Germany) and stained with uranyl acetate and lead citrate, and then observed under an electron microscope (JEM 1200EXII, Tokyo, Japan).

Results

In the NTA-treated groups (groups A-7 and B-4) there were no obvious changes in the livers or in the kidneys. In the Fe-NTA-treated groups (groups A-1–A-6 and B-1–B-3), the hepatocytes, Kupffer cells and some endothelial cells in the peripheral area of the hepatic lobules contained iron depositions that were stained by Perls' method. The iron depositions were more abundant in the peripheral areas than in the central areas of the liver (Fig. 1A). In some kidneys, haemosiderin was observed in the cytoplasm of some proximal and distal tubules.

In many liver parenchymal cells from animals in groups A-1–A-6, B-1, and B-3, iron depositions were observed not only in the cytoplasm but also in the nuclei. The nuclear depositions were spherical and the size of large nucleoli (Fig. 1B). Some hepatocytes showed intranuclear iron depositions but not intracytoplasmic iron depositions (Fig. 1C). In the sections rinsed with acidic solution for 120 h much of the cytoplasmic iron deposition had decreased, whereas the intranuclear iron deposition persisted (Fig. 1D). Intranuclear iron depositions were also found in some kidneys of animals in both A and B groups. They were smaller than those in the livers, and mostly only one deposition was observed in each nucleus (Fig. 1E). In the renal tubules, cytoplasmic and nuclear iron depositions were less often found than in hepatocytes (Tables 1, 2). There was no significant difference in the incidence of iron depositions in the liver and kidney between male and female animals, as shown by the cumulative Chi-square test. In the B groups, most of the intranuclear iron depositions were observed in the animals treated with Fe-NTA for 12 weeks and sacrificed at 1 year after Fe-NTA treatment. The incidence of intranuclear iron deposition in the livers of the group B-1 mice was significantly higher than in those of the other groups according to the cumulative Chi-square test. The incidence of intranuclear iron deposition in the

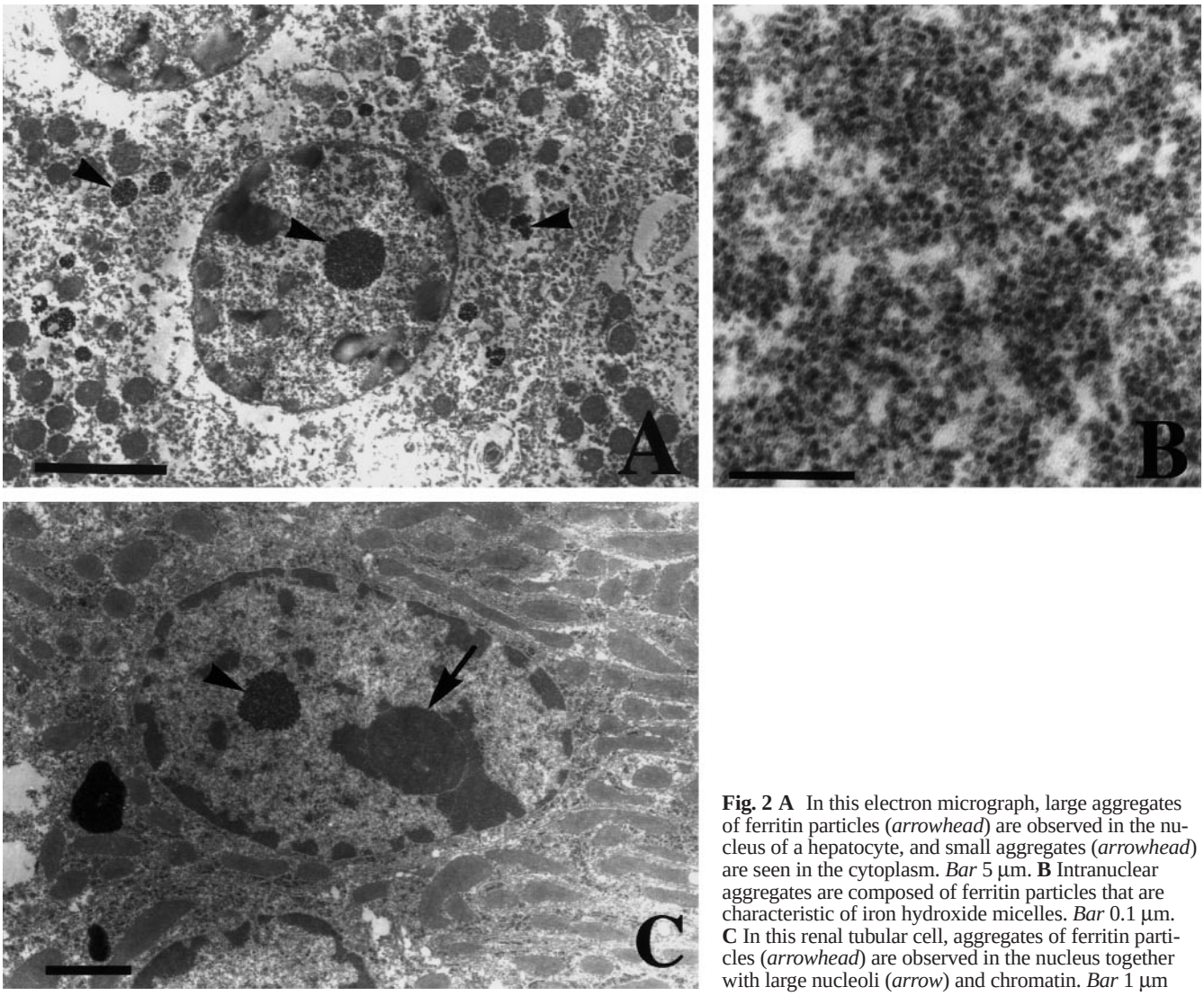

Fig. 2 A In this electron micrograph, large aggregates of ferritin particles (*arrowhead*) are observed in the nucleus of a hepatocyte, and small aggregates (*arrowhead*) are seen in the cytoplasm. *Bar* 5 μm. **B** Intranuclear aggregates are composed of ferritin particles that are characteristic of iron hydroxide micelles. *Bar* 0.1 μm. **C** In this renal tubular cell, aggregates of ferritin particles (*arrowhead*) are observed in the nucleus together with large nucleoli (*arrow*) and chromatin. *Bar* 1 μm

animals examined at 12 months after the treatment was generally higher than that in the clinically unwell animals examined at 1 month or several months after the treatment. The location and incidence of iron depositions in the animals of each group are summarized in Tables 1 and 2. We found no intranuclear iron deposition in the animals treated with Fe-NTA for 1 or 2 weeks. The number of animals positive for iron in each organ rose with increasing Fe-NTA administration. There was much variation in the number of positive cells per sections of each organ. Livers had more positive cells than kidneys.

Twelve months after the first treatment with Fe-NTA, renal tumours (Fig. 1F) were found in several animals. The incidence was 19% in group A-1, 29% in group A-6 and 8% in group B-1.

The iron depositions in the nuclei of hepatocytes were strongly positive for ferritin but those in the cytoplasm were weakly and granularly positive (Fig. 1G). In the kidneys, the cytoplasmic staining for ferritin was weak and granular, but strong staining for ferritin was observed in the nuclei (Fig. 1H).

Electromicroscopic observation revealed that the large round intranuclear iron depositions were devoid of any boundary membrane (Fig. 2A). The individual particle size of approximately 10 nm was characteristic of the iron hydroxide micelles of ferritin (Fig. 2B). In the renal tubular cells, intranuclear iron aggregates and large nucleoli were also observed (Fig. 2C). The diameters of the iron deposits were 2.2–2.6 μm in the liver and 0.6–1.0 μm in the kidney.

Discussion

We observed intranuclear deposition of iron–ferritin complexes in the Fe-NTA-treated animals, revealed by Perls' iron staining, immunohistochemistry and electron microscopy. Our findings are consistent with the observations of intranuclear ferritin inclusions after iron loading with some iron complexes by Haddow and Horning [15], Goetz and Richter [12] and Smith et al. [29]. Perls' iron staining and immunohistochemistry showed round and aggregated iron depositions consisting of ferritin particles in the nuclei, while only granular and nonaggregated iron depositions were observed in the cytoplasm. In 50–70% of cells in one section of a liver we observed detectable iron deposition only in the nuclei and not in the cytoplasm in five animals. We speculate that cytoplasmic iron was mobilized and utilized, while intranuclear iron could not be used. In acidic soultion, cytoplasmic iron was dissolved, but intranuclear iron was not dissolved. It is possible that the intranuclear iron was in a hydrophobic environment because the iron aggregate was precisely spherical (the form with the smallest volume) and was without a membrane and resistant to acidic iron mobilization.

The ferritin molecule may have passed from the cytoplasm into the nuclei in the Fe-NTA-treated animals. The protein shell of horse spleen ferritin has an external diameter of 12–13 nm and a cavity 6–9 nm in diameter [6]. The cellular nucleus has pores, and molecules less than 10 nm in diameter pass through the pores by passive diffusion. Larger molecules require an energy-dependent transport mechanism [11]. Ferritin may thus pass through the nuclear membrane by means of some energy-dependent transport system. Smith et al. indicated that ferritin can pass with a slow but finite diffusion; they found different concentrations of ferritin particles in the nucleus and cytoplasm [29]. Cai et al. have set up the hypothesis that ferritin becomes associated with another molecule that has a nuclear localization signal (NLS), since it is unlikely that a conventional nuclear localization signal is involved in the nuclear translocation of the ferritin [8].

Low-molecular-weight iron can easily pass through the nuclear membrane. It is reported that the nuclei of rat liver cells take up iron from ferric citrate by a process that is dependent on adenosine 5′-triphosphate (ATP) [13]. Another possible mechanism of ferritin deposition in the nucleus is separate entry of iron and the ferritin subunit into the nucleus with incorporation of iron into the ferritin particle in the nucleus.

The administration of Fe-NTA stimulates oxidative damage to DNA and induces the formation of 8-hydroxydeoxyguanosine in rat kidneys [31, 32]. The interaction of iron with DNA in the nucleus is necessary for the oxidative damage. In the present study, the presence of iron in the nucleus was confirmed. If free iron is released in the nucleus, the iron-catalysed free radical reaction might generate reactive oxygen species, which might then cause oxidative damage to DNA that would induce renal tumours. It has been reported that hepatocellular carcinoma (HCC) develops in about 30% of haemochromatotic patients and that the relative risk of HCC in patients with haemochromatosis and cirrhosis is over 200-fold that in patients without haemochromatosis [7, 23].

Lipid peroxidation may also play an important part in carcinogenesis by iron [24] and was incuded at 30–90 min after the injection of Fe-NTA in mice [19, 26]. The incidence of renal tumours and the extent of renal tubule lipid peroxidation were greater in male than in female rats and mice [10, 18, 19, 26, 30]. Several renal tumours were observed in male animals and in ovariectomized and testosterone-treated female animals in the present experiment; iron-induced free radicals in the nucleus may have played a part in this as a promoter. In the present experiment there was no difference in the amount of iron deposition between male and female animals. However, in another study lipid peroxidation was slight and tumour development was not observed in livers of animals treated with Fe-NTA [24].

Ferritin traps iron and prevents the peroxidative effects of iron-induced free radicals. More intranuclear ferritin was found in the hepatocytes than in the renal cells in the present study, suggesting that ferritin prevents the iron-mediated generation of free radicals and plays some part in prevention of hepatic tumour development.

The intranuclear iron depositions were more abundant in those of our animals that were treated with Fe-NTA

for 12 weeks than in those treated for 4 weeks or 8 weeks. Thus, repeated injections of Fe-NTA elevated the level of cytoplasmic ferritin. It has been reported that oxidative stress induces ferritin synthesis [9]. It is likely that iron-mediated oxidative stress promotes cytoplasmic ferritin synthesis and causes ferritin accumulation in the nuclei. Ferritin may then aggregate gradually after passing through the nuclear membrane. Studies of intranuclear ferritin accumulation will elucidate the mechanism of iron-mediated free radicals and renal carcinogenesis induced by Fe-NTA.

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