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Vitamin E Inhibits Apoptosis, DNA Modification, and Cancer Incidence Induced by Iron-mediated Peroxidation in Wistar Rat Kidney[1]

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ABSTRACT

We have developed an experimental model of iron-induced oxidative nephrotoxicity and renal cancer. Using this model, the effect of vitamin E, a known antioxidant, was investigated. Three-week-old male Wistar rats were fed with vitamin E-sufficient (control) and vitamin E-supplemented diets throughout the experiment. After 1 month of feeding, iron-induced tissue lipid peroxidation, apoptosis, and formation of 8-hydroxydeoxyguanosine, a known DNA oxidative modification, were observed by cold Schiff staining, *in situ* labeling method (staining by terminal deoxynucleotidyl transferase-mediated nick end labeling), and high-performance liquid chromatography with electrochemical detection system, respectively, in the groups of rats treated with ferric nitrilotriacetate (Fe-NTA; Fe, 10 mg/kg body weight). For the vitamin E intervention study on Fe-NTA-induced renal carcinogenesis, two groups of rats fed vitamin E-sufficient and vitamin E-supplemented diets (30 and 20 rats, respectively) were treated with Fe-NTA (Fe, 7.5 mg/kg body weight once or twice a week) i.p. for 3 months and observed for 9 additional months. Five of the vitamin E-sufficient rats died during the first 3-month period. The results showed that vitamin E could inhibit tissue lipid peroxidation, apoptosis, 8-hydroxydeoxyguanosine formation, and the development of cancer [11 of 25 rats (44%) for vitamin E-sufficient versus 1 of 20 rats (5%) for vitamin E-supplemented rats, respectively]. These studies strongly suggest that in Fe-NTA-induced renal cancer, as with certain other types of cancer, oxidative stress plays an important role in carcinogenesis, and an antioxidant is an effective chemopreventive measure.

INTRODUCTION

It has been reported that oxidative stress can induce DNA damage, such as DNA fragmentation and apoptosis (1), base modifications (such as thymine dimers or 8-OHdG[3] formation; Ref. 2), and DNA strand breaks (3). Oxidative modification of DNA is thought to be implicated in mutagenesis and carcinogenesis (1, 4). Kuchino *et al.* (5) and Shibutani *et al.* (6) showed that 8-OHdG causes G-to-T transversions. Many studies have confirmed that 8-OHdG is a good marker that reflects free radical-mediated DNA modification (7, 8).

Some antioxidants, including vitamin E (α-tocopherol), have been reported to protect DNA from fragmentation (9) and to inhibit cancer incidence in various organs of experimental animals (10–13), but definitive evidence for the beneficial effect of vitamin E is still disputed (14). To date, no studies have shown that antioxidants inhibit 8-OHdG generation induced by oxidative damage *in vivo*, probably because there are few experimental models of tissue damage and cancer induction by oxidative stress.

We have developed a model of iron-induced oxidative tissue damage and carcinogenesis using Fe-NTA and ferric ethylenediaminediacetate in rats and mice (15), and it was shown that 8-OHdG formation was elevated after Fe-NTA injection in rats (16–18). To provide evidence for the role of radical reactions in cancer induction, it was, therefore, important to see if an antioxidant could modify the 8-OHdG generation, other markers of oxidative damage, and finally, cancer incidence induced by the iron chelate. In this study, we report the inhibitory effect of vitamin E on iron-mediated, free radical-induced apoptosis, 8-OHdG generation, and the incidence of renal cancer. These studies suggest that vitamin E may provide chemoprevention in types of oxidative stress-induced cancer.

MATERIALS AND METHODS

Chemicals. Vitamin E-sufficient (control) diet (α-tocopherol, 2.0 units/100 g) and vitamin E-supplemented diet (α-tocopherol, 50.0 units/100 g) were supplied by Eisai Pharmaceutical Co. (Tokyo, Japan). Biotin-16-2′-deoxyuridine-5′-triphosphate was obtained from Boehringer Mannheim (Mannheim, Germany). Terminal deoxynucleotidyl transferase and all other reagents were of the highest quality available from Wako Pure Chemicals, Inc. (Osaka, Japan). Fe-NTA was prepared by mixing ferric nitrate solution with nitrilotriacetic acid disodium salt solution (15).

Animals. Male Wistar rats (3 weeks old) obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were used.

Eighty rats were divided into two groups. One group (45 rats) was fed with the vitamin E-sufficient diet, and the other group (35 rats) was fed with the vitamin E-supplemented diet throughout the experiment. At the end of the 1st month, five rats from each group were killed to determine α-tocopherol levels in sera, livers, and kidneys. Measurement of α-tocopherol was done by courtesy of Eisai Pharmaceutical Co.

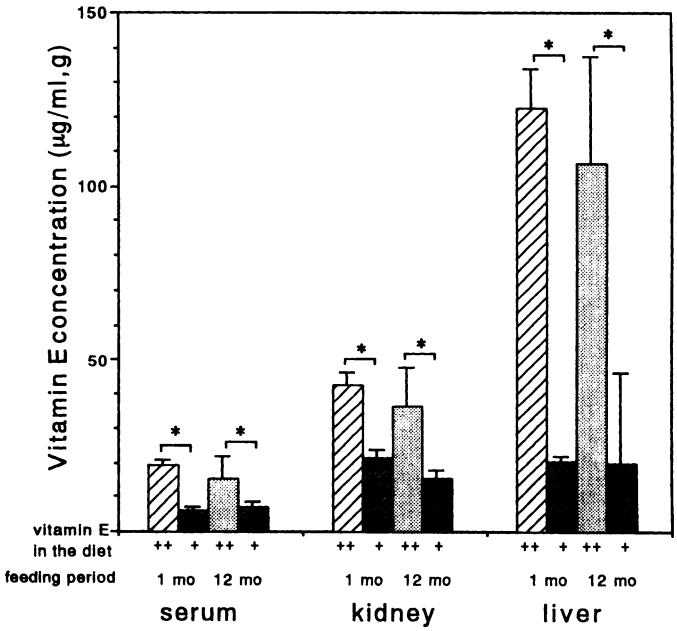

Fig. 1. The vitamin E concentration in male Wistar rats. Vitamin E concentration was determined just before Fe injection (1 month of feeding) and at the end of the experiment (12 months of feeding). *Columns*, mean concentrations of vitamin E in various tissues of 10 rats fed a vitamin E-supplemented (++) and 10 rats fed a vitamin E-sufficient diet (+); *bar*, SD. *, $P < 0.01$ (Student's *t* test).

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[3] The abbreviations used are: 8-OHdG, 8-hydroxydeoxyguanosine; Fe-NTA, ferric nitrilotriacetate; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

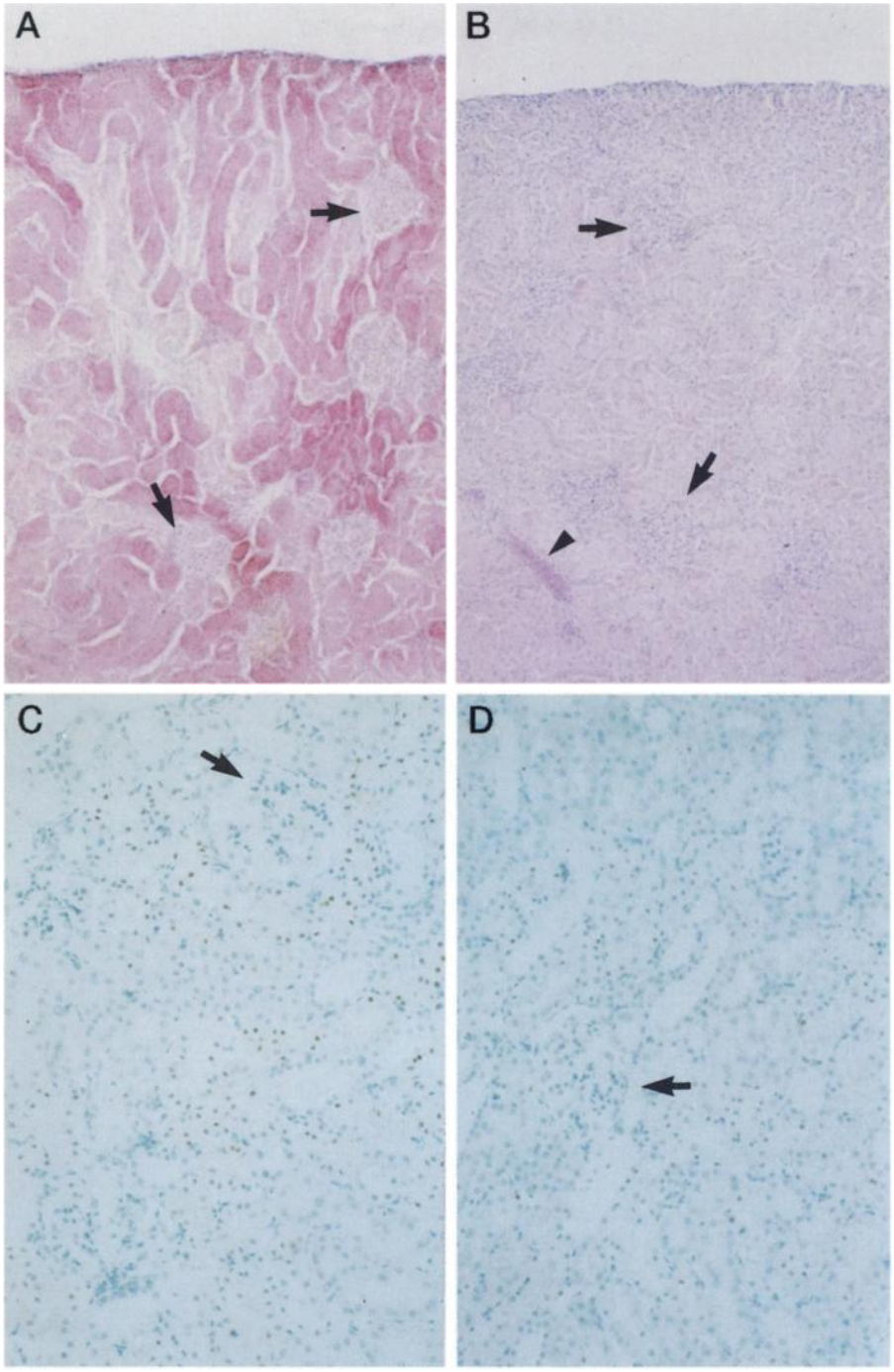

Fig. 2. *A*, the iron-induced lipid peroxidation in the kidney of rat with vitamin E-sufficient diet. The sample was taken 1 h after Fe-NTA injection (Fe, 10 mg/kg body weight) i.p. Strongly positive staining in proximal tubules is shown. *Arrow*, glomerulus. Cold Schiff staining. Magnification, ×100. *B*, same treatment as in *A* in rats fed with vitamin E-supplemented diet. Only lightly positive staining in proximal tubules is observed. The positivity in vascular wall (*arrowhead*) is normal. *Arrow*, glomerulus. Cold Schiff staining. Magnification, ×100. *C*, *in situ* end labeling (TUNEL staining) in renal tissue of a rat fed with a vitamin E-sufficient diet and receiving Fe-NTA injection (Fe, 10 mg/kg body weight) i.p. The sample was taken 1 h after injection. TUNEL-positive nuclei are in the proximal tubules. *Arrow*, glomerulus, TUNEL staining. Magnification, ×100. *D*, same treatment as in *C* in the rat fed vitamin E-supplemented diet. Only a few TUNEL-positive cells are observed. *Arrow*, glomerulus. TUNEL staining. Magnification, ×100.

Experimental Procedures. After 1 month of feeding, 10 rats from each group were treated i.p. with Fe-NTA (Fe, 10 mg/kg body weight) and then killed after 0, 1, 6, and 24 h. The kidneys were taken out, half of the tissues were frozen at −80°C for cold Schiff staining and 8-OHdG determination, and the remaining halves were fixed with 10% neutral formalin, embedded in paraffin, and cut into thin section for routine H&E and TUNEL staining (18).

Cold Schiff staining (19) was used for observing the lipid peroxidation in renal tissue of the rats (20–22). Peroxidized areas were stained purple by this method.

The DNA extraction from kidney tissue and determination of 8-OHdG were carried out according to method of Kasai *et al.* (23) by using DNA Extractor WB Kit (Wako Pure Chemicals, Inc.) and a high-performance liquid chromatography with electrochemical detector system (Waters LC Module I, Millipore, Japan, Tokyo, Japan; esa Coulochem II, ESA Inc., Bedford, MA).

In situ end-labeling method (TUNEL staining; Ref. 24) was used for confirming the apoptosis of histological specimens from rat kidney.

The remaining 30 of the vitamin E-sufficient and 20 of the vitamin E-supplemented rats were used for a study of chemoprevention of Fe-NTA-induced renal cancer. Both groups of rats were injected with Fe-NTA (Fe, 7.5 mg/kg body weight, once or twice a week) for 3 months and kept untreated for another

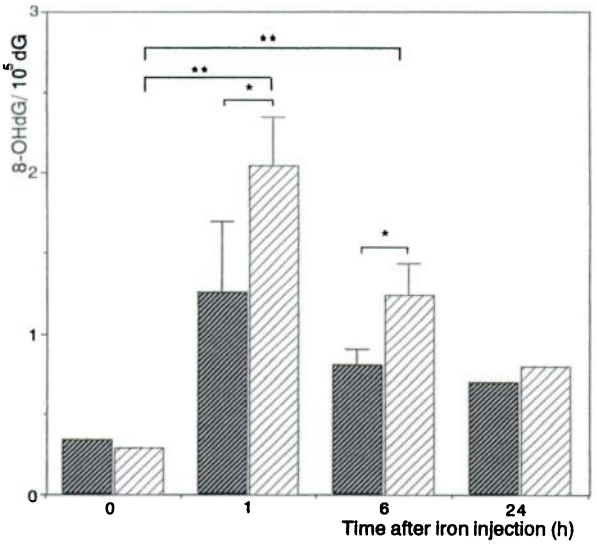

Fig. 3. 8-OHdG formation in rat kidneys after Fe-NTA injection (Fe, 10 mg/kg body weight) i.p. Male Wistar rats were fed either with vitamin E-sufficient diet (▨) or vitamin E-supplemented-diet (■) for 1 month as described in "Materials and Methods." 8-OHdG was determined by high-performance liquid chromatography with electrochemical detection. Shown is the ratio of 8-OHdG relative to deoxyguanosine (*dG*) in the rat kidney DNA. As 8-OHdG at 0 h appears not to be different between vitamin E-sufficient ($n = 2$) and -supplemented rats ($n = 2$) based on the average of two determinations, two groups were combined ($n = 4$) for the statistical analysis at 0 and 24 h ($n = 2$) and at 1 and 6 h ($n = 3$), *, $P < 0.05$ and **, $P < 0.01$; (two groups combined at 0 h versus groups of different vitamin E status at 1 and 6 h, Student's *t* test). *Columns*, means; *bars*, SD.

9 months. At the end of the experiment, all the rats were killed under ether. The sera, livers, and kidneys were taken out for measuring α-tocopherol level (Fig. 1), and liver and kidney tissues were fixed with 10% neutral formalin and processed for routine histological observations by H&E stain.

RESULTS

Demonstration of Lipid Peroxidation by Cold Schiff Staining. The renal parenchyma of the untreated normal rats was completely unstained after exposure to Schiff reagent (data not shown). However, arterial walls were positively stained (see Fig. 2*B*, *arrowhead*). Strongly positive staining was seen in specimens from Fe-NTA-injected rats fed a vitamin E-sufficient diet (Fig. 2*A*). Only lightly positive staining was found in rats fed a vitamin E-supplemented diet (Fig. 2*B*).

Determination of 8-OHdG. The 8-OHdG levels in the rat kidney DNA increased significantly at 1 and 6 h after Fe-NTA injection in both vitamin E-sufficient and -supplemented groups. The level was higher in the vitamin E-sufficient group, compared with that in the vitamin E-supplemented group at 1 and 6 h after Fe-NTA injection (Fig. 3).

Apoptosis by TUNEL Staining. Positive staining nuclei were numerous in the outer stripe of the outer medulla of rats from vitamin E-sufficient group at 1 h after Fe-NTA injection, but only a few positive nuclei were seen in kidneys from the vitamin E-supplemented group (Fig. 2 *C* and *D*).

Cancer Incidence. The results of α-tocopherol levels determined in sera, kidneys, and livers of rats fed with vitamin E-sufficient and -supplemented diet before iron injection and at the end of the experiment are shown in Fig. 1. Animals fed with vitamin E-supplemented diet kept higher weight than animals fed with vitamin E-sufficient diet during and after iron injection (Fig. 4). Five of the vitamin E-sufficient rats died during the first 3 months.

Eleven cancers were found in the 25 vitamin E-sufficient rats that survived the 3-month treatment, for a frequency of 44%. Cancer occurred in only 1 of 20 rats (5%) in the vitamin E-supplemented group. The incidence of cancer was significantly ($P < 0.01$) different between the two groups. A comparison of the histological observations on renal tissue injury between both groups is shown in Fig. 5*A* and *B*. Severe cystic lesions occurred in vitamin E-sufficient group, as compared with vitamin E-supplemented group. A typical adenocarcinoma occurring in renal tissue in the vitamin E-sufficient group is shown in Fig. 4 *C* and *D*.

DISCUSSION

Fe-NTA induces a high incidence of renal cancer in rats and mice (15). There is increasing evidence that free radicals play an important role in cancer induction of this iron complex (15, 22, 25). The cytoplasmic location for the lipid peroxidation has been well visualized by cold Schiff stain and by demonstration of 4-hydroxynonenal-protein complex, a lipid peroxidation product (20–22). In the present work, positive cold Schiff staining was found in the proximal tubules of kidney tissue of rat with vitamin E-sufficient diet at 1 h after Fe-NTA injection (Fig. 2*A*).

Apoptosis is a morphologically distinct type of cell death that is also induced by oxidative damage (26). The apoptotic cells induced by Fe-NTA were observed by TUNEL, as well as by H&E staining. Increased positive TUNEL staining was found in cell nuclei of proximal tubules of kidney tissue from vitamin E-sufficient diet group at 1 h after Fe-NTA injection (Fig. 2*C*). Randomly scattered cells with pyknotic nuclei and shrunken eosinophilic cytoplasm, an indication of apoptotic cells, were observed by H&E stain at the same time (15). Severe cell death that belonged to renal proximal tubules was seen in specimens from vitamin E-sufficient group at 24 h after Fe-NTA injection (15). To distinguish apoptotic cells from necrotic cells in the 24-h specimen was impossible. We recently reported the iron-induced free radicals may be responsible for the apoptosis (27).

The most ubiquitous oxidative DNA base modification is 8-OHdG (1, 2, 8). In the present study, a high 8-OHdG level appeared at a time of elevated TUNEL staining in the vitamin E-sufficient diet rats. These findings are consistent with the earlier *in vitro* observation that severe oxidative DNA damage (DNA strand fragmentation and base modification) had occurred in Fe-NTA-treated DNA (3, 17, 28). In contrast, only a few lightly positive Schiff-staining cells, a few positive TUNEL-staining cells, and a low level of 8-OHdG appeared in

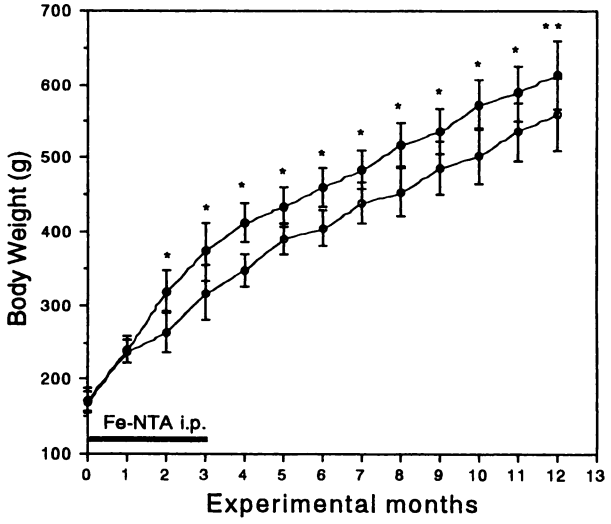

Fig. 4. Body weight of rats treated with Fe-NTA for 3 months and kept observed for 9 months. ○, vitamin E-supplemented; ●, vitamin E-sufficient diet. *, $P < 0.01$; **, $P < 0.05$, relative to vitamin E-sufficient rats (Student's *t* test).

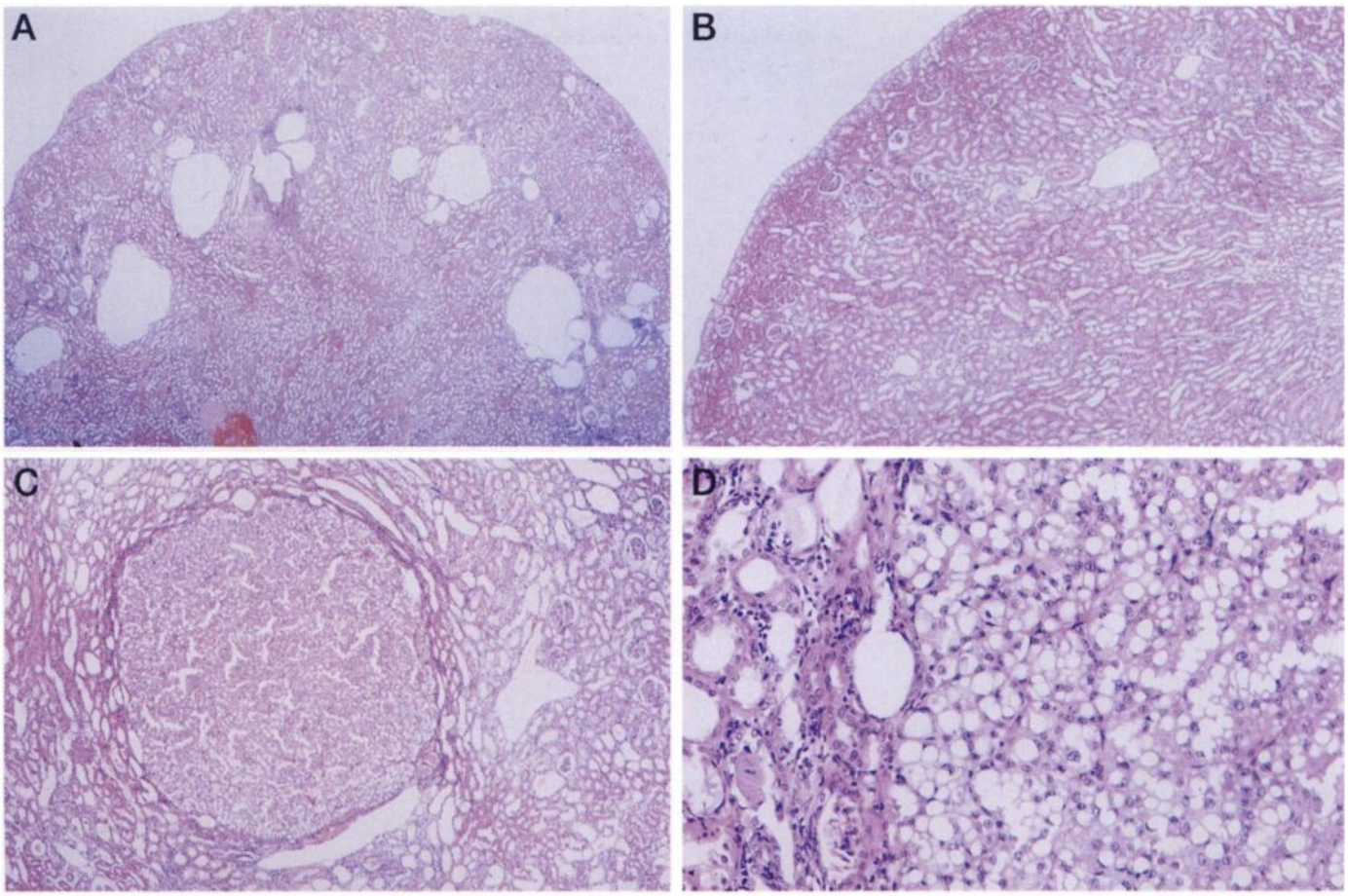

Fig. 5. Tumor histology. *A*, a microscopical picture of a non-tumor-bearing kidney induced by Fe-NTA (Fe, 7.5 mg/kg body weight, i.p., one or two times a week) for 3 months in a rat fed with a vitamin E-sufficient diet. The rat was killed 9 months after last Fe-NTA injection. There are numerous cyst formations in the outer stripe of outer medulla, where lipid peroxidation was the severest after Fe-NTA injection. H&E staining. Magnification, 40×. *B*, same treatment as in *A* in a rat fed with vitamin E-supplemented diet. Morphological alteration is minimal. H&E staining. Magnification, ×400. *C*, a typical histological view of a renal cancer seen in a rat treated as in *A*. H&E staining. Magnification, ×40. *D*, a magnification of *C*. H&E staining. Magnification, 200×.

samples from vitamin E-supplemented group. This result is consistent with our previous report (29), which indicated that iron-induced lipid peroxidation by Fe-NTA was inhibited in kidneys of vitamin E-supplemented rats.

In conclusion, it is shown that, in our model of iron-induced renal carcinogenesis, free radicals play an important role in cancer induction. Obvious differences in cancer incidence were found between experimental animals on a vitamin E-sufficient diet and those with vitamin E-supplemented diet. These facts suggest that vitamin E as an antioxidant is an effective chemopreventive measure against the type of carcinogens in which free radicals are involved in the induction of cancer.

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