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ORIGINAL ARTICLE

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Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and artepillin C

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Abstract In experiments using the renal carcinogen ferric nitrilotriacetate (Fe-NTA) in male ddY mice, primary pulmonary cancers were also induced in bronchiolar and alveolar tissues. 4-Hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2'-deoxyguanosine (8-OHdG), products of oxidative processes, increased in bronchiolar and alveolar cells after administration of Fe-NTA. These substances disappeared after oral administration of propolis or artepillin C, as shown histochemically, and correlated with an anticancer prophylactic effect of propolis and artepillin C. From our investigation, lipid peroxidation seems to play an important role in pulmonary carcinogenesis. Malignant progression from adenoma of bronchiolar or alveolar origin to malignant tumors has been proposed to involve a stepwise transformation. In our study, adenomas developed into adenocarcinomas and large cell carcinomas after treatment with Fe-NTA. In contrast, after oral administration of propolis or artepillin C, adenomas did not progress to carcinomas. Instead of developing into large cell cancers, as induced by Fe-NTA in control mice, adenomas showed remarkable proliferation of macrophages and local anti-oxidant activity after treatment with either propolis or artepillin C. Propolis and artepillin C therefore appear to inhibit lipid peroxidation and the development of pulmonary cancers.

Keywords Ferric nitrilotriacetate · Lipid peroxidation · Pulmonary tumors · Propolis · Artepillin

Introduction

In an effort to better understand carcinogenesis in human tissues, investigators have developed animal models of the disease and have applied a variety of agents to prevent carcinogenesis. Several studies concerning the development of mouse lung adenomas [2, 9, 11, 12, 13, 27, 42] have revealed two major histologic types of cancers, one with alveolar and the second with bronchiolar or papillary characteristics. Determination of the origin of these cancers, from alveolar or bronchiolar epithelia, is largely resolved using the electron microscopic identification of alveolar type-II cells in adenomas [4, 6, 26, 39]. Biochemical studies on phospholipid synthesis in adenomas have shown similarities to type-II cells [28, 43]. Thus, the origin of histologically different types of cancers from alveolar epithelium became widely accepted [41]. Kennedy et al. [21] showed that the first experimentally produced cancerous Clara cells share some features in common, but they are distinct ultrastructurally and histochemically [8, 16, 19, 20]. Clara cell hyperplasia has been induced in mice by chronic exposure to urethane [44] and produced in rats by repeated exposure to 4-nitroquinoline-1-oxide [31]. Thus, there is ample evidence that Clara cells are capable of both hyperplastic and neoplastic conversion [38].

Injection of iron-chelated nitrilotriacetate (Fe-NTA) causes severe acute and renal proximal tubular necrosis through Fe-induced free radical chemistry [18, 34, 35]. Eventually, the chemical induces renal cancers in rats and mice that survive nephrotoxicity [14, 15, 33, 35, 46, 51]. In a recent study, we examined the induction of renal cancer by Fe-NTA and found that carcinogenesis of the renal proximal tubular epithelium was induced by oxidative damage and was followed by renal carcinoma [25]. In the same experiments, propolis, a product of bees, and artepillin C, a component of propolis, exhibited inhibitory effects on renal carcinogenesis as a result of their oxyradical scavenging properties. We have noticed that mice given Fe-NTA also developed signs of neoplastic tissues in the lungs. Investigations revealed that these were primary lung cancers, that they were originating from alveolar and bronchiolar cells, and that they were not metastasis of renal origin. Histological and histochemical analysis showed that carcinogenesis in the lungs significantly correlated with the extent of pulmo-

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nary lipid peroxidation. Oral administration of either propolis or artepillin C protected the tissues from oxidative damage and inhibited carcinogenesis in the mice. This is the first report showing that Fe-NTA, a known renal carcinogen, can also produce tumors of the lungs and that its carcinogenic effects in multiple organs can be inhibited by propolis and artepillin C.

Materials and methods

Chemicals

NTA disodium salt and 2,6-di-tert-butyl-p-cresol were purchased from Nacalai Tesque (Kyoto, Japan). 4-Hydroxy-2-nonenal (4-HNE) modified protein and 8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody were purchased from Nikken Food Co., Ltd. (Shizuoka, Japan). Proliferating cell nuclear antigen (PCNA, PC10) antibody was purchased from Santa Cruz Biotechnology Inc. (Calif.). Thyroid transcription factor-1 (TTF-1) antibody, peroxidase-conjugated rabbit anti-mouse immunoglobulins, and ABC complex (avidin and biotinylate peroxidase) were purchased from Dako (Glostrup, Denmark). All other chemicals used in this study were purchased from Wako Fine Chemicals (Osaka, Japan).

Fe-NTA was prepared immediately before use by the method of Awai et al. [3]. Briefly, ferric nitrate and NTA disodium salt were both dissolved in deionized water. These were mixed at a molar ratio of 1:4 at room temperature, and the pH was adjusted to 7.4 with sodium bicarbonate. Artepillin C was extracted from Brazilian propolis according to Aga's method [1]. The chemical name of artepillin C is 3-[4-hydroxy-3, 5-bis (3-methyl-2-butenyl) phenyl]-2-propenoic acid, but it is commonly referred to as 3,5-diprenyl-4-hydroxycinnamic acid. The chemical formula of artepillin C is $C_{19}H_{24}O_3$, and its molecular weight is 300.40. Because artepillin C goes poorly into solution in water and, since the artepillin C used in this investigation was to be used in solution, the substance was first dissolved in 1 ml of absolute ethanol and then diluted with distilled water. The liquid form of Brazilian propolis used in this study was in solution in ethanol and contained water-soluble levels of artepillin C, flavonoids, and prenylcaffeic acid.

Animals

Male ddY mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and kept under quarantine for 1 week before starting the experiments. The animals were maintained on a diet of commercial mouse chow (Oriental Yeast Co., Tokyo, Japan) and tap water ad libitum.

Evidence of carcinogenesis

Experiment 1

The mice were divided into four groups. These were untreated, treated with propolis at 1.0 mg/mouse, treated with artepillin C at 100 µg/mouse, or treated with 5% ethanol (vehicle only) as the artepillin C control group. Animals of all four groups were injected with Fe-NTA at a dose of 10 mg of Fe/kg per day, i.p. twice per week for 8 weeks. Samples were given to the mice twice per day (5 days/week) throughout the experiment. All surviving animals were sacrificed under ether anesthesia 12 months after the last FeNTA treatment.

Experiment 2

Four-week-old male ddY mice were used to confirm the results in experiment 1. Mice were divided into four groups. These were treated with propolis (1.0 mg/mouse) in 1% ethanol or 1% ethanol only as its control group, and treated with artepillin C at 350 µg/mouse or olive oil as its control group. All animals were injected with Fe-NTA at a dose of 10 mg of Fe/kg per day, i.p. twice per week for 8 weeks. Propolis in 1% ethanol was given to the mice for 5 days per week, and artepillin C in olive oil was given to the mice twice per day (5 days/week) throughout the experiment. All surviving animals were sacrificed under ether anesthesia 12 months after the last Fe-NTA treatment. Lungs were horizontally sectioned and fixed in 10% phosphate-buffered formalin and embedded in paraffin. These were stained with periodic acid-Schiff reagent (PAS), Berlin blue, and hematoxylin and eosin (H&E) staining.

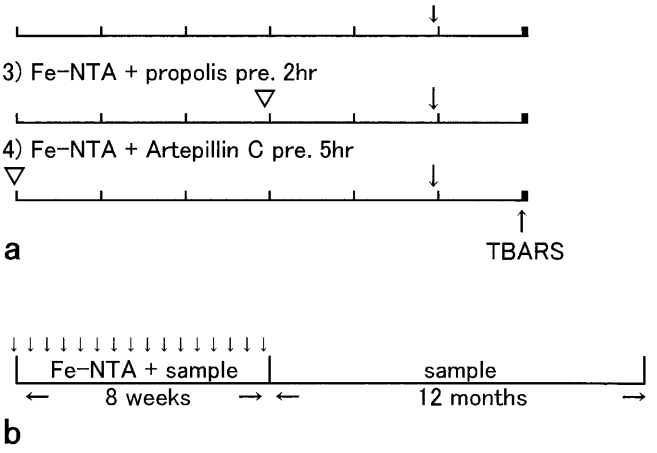

Fig. 1 Administration protocol for the induction of acute nephrotoxicity (**a**) and carcinogenesis (**b**). **a** Ferric nitrilotriacetate (FeNTA) (↓), 7 mg Fe/kg were given by means of i.p. injection; propolis or artepillin C were administered by means of gastric intubation (▽). Mice were sacrificed 1 h after Fe-NTA injection or treatment, and levels of thiobarbituric acid-reactive substances (TBARS) were measured (△). **b** Fe-NTA(↓), 10 mg Fe/kg was given by means of i.p. injection. In experiment 1, EtOH-water, propolis, and artepillin C were given orally throughout the experiment. In experiment 2, EtOH-water and propolis were given in drinking water, and olive oil and artepillin C were given orally throughout the experiment. Mice were sacrificed 12 months after the final Fe-NTA injection

Acute toxicity

Eight-week-old male ddY mice were used in these experiments. The mice were given propolis (1.0 mg/mouse in 1% ethanol) or artepillin C (100 µg/mouse in 1% ethanol) by the oral route at 2 h or 5 h before i.p. injection of Fe-NTA at a dose of 7 mg Fe/kg (Fig. 1a). This schedule had been shown to protect mice from renal toxicity induced by Fe-NTA [25]. From the results of absorption of artepillin C, the maximal absorption in the organs was found to be 6 h after oral administration. Therefore, in this experiment, propolis and artepillin C were administered 2 h or 5 h before injecting Fe-NTA. The mice were sacrificed under anesthesia 1 h after injecting Fe-NTA. A lobe from the lung was removed and chilled in ice-cold physiological saline, and homogenized in 1.15% KCl for the measurement of thiobarbituric acid-reactive substances (TBARSs). Levels of TBARSs were determined using the fluorimetric method of Ohkawa et al. [32]. Another lobe from the lung was rinsed with cold phosphate-buffered saline (PBS), quickly frozen, and stored at –80°C. Samples were treated with cold Schiff's reagent according to the method of Pompella et al. for histochemical determination of peroxidation [37]. Another lobe of the lung was fixed in Bouin's solution and embedded in paraffin for immunohistochemistry, PAS, and H&E staining.

Table 1 Incidence of tumors in male ddY mice injected with FeNTA alone or in mice receiving Fe-NTA with either propolis or artepillin C pre-treatment. In experiment 1, 5% EtOH was administered as the control vehicle for both propolis and artepillin C. In experiment 2, 1% EtOH solution and olive oil were administered as the control vehicles for propolis and artepillin C, respectively

Treatment group	Assigned	Surviving throughout	Number of mice with lung disorder	
			Adenoma	Adenocarcinoma
Experiment 1				
Untreated	7	7	1	0
Fe-NTA-treated				
5% EtOH	13	4	1	2
Propolis	13	10[a]	3	0
Artepillin C	12	6	2	0
Experiment 2				
Fe-NTA-treated				
1% EtOH	12	8	1	4
Propolis	12	9	1	0[b]
Olive oil	12	9	1	4
Artepillin C	12	6	2	0[c]

[a] Significant difference from the 5% EtOH group by Fisher's exact probability test ($P<0.05$)
[b] Significant difference from the 1% EtOH group by Fisher's exact probability test ($P<0.05$)
[c] Significant difference from the olive oil group by Fisher's exact probability test ($P<0.05$)

Immunohistochemistry

The indirect method for immunohistochemistry was used to determine 4-HNE, 8-OHdG, and PCNA. After deparaffinization with xylene and ethanol, incubation in 3% hydrogen peroxide in PBS was applied for the inhibition of endogenous peroxidase. After these procedures, sections on slides were stained with normal rabbit serum for the inhibition of non-specific binding of secondary antibody, partially purified mouse antibody against 4-HNE-modified proteins (0.5–2.0 µg/ml) [5, 36, 47, 49, 52], 8-OHdG (10 µg/ml) [10, 36, 40, 48, 50], or PCNA (0.4 µg/ml) according to standard procedures. Peroxidase-conjugated rabbit anti-mouse immunoglobulins were then applied to the sections. Control normal rabbit serum instead of antibodies against either 4-HNE-modified proteins or 8-OHdG formation in DNA showed only weak positivity. The ABC method for immunohistochemistry was used for detecting TTF-1 staining [17]. A rabbit polyclonal antiserum against thryoglobulin at an appropriate dilution was used to stain paraffin sections after these were pretreated in a pressure cooker. Sections were then treated sequentially with biotinylated anti-rabbit immunoglobulin (Ig)G, ABC complex, and dimethylaminoazobenzene.

Statistical analysis

Statistical analysis was carried out using the Student's *t*-test for differences in the levels of TBARSs and the Fischer's exact test for differences in tumor incidences.

Results

Carcinogenesis

Table 1 shows the incidence of Fe-NTA-induced adenomas and adenocarcinomas in untreated or treated mice and their survival in two experiments. A high incidence of lung adenocarcinomas in mice injected with Fe-NTA was observed 1 year later in the animals that survived nephrotoxicity. However, the mice administered low doses of propolis or artepillin C orally appeared to enjoy partial protection from developing malignant cancers in experiment 1. To confirm our results, we performed experiment 2, where we treated the mice with higher doses of artepillin C. In this experiment, a significant protection from the development or progression of adenomas to adenocarcinomas was observed in mice treated with propolis or artepillin C ($P<0.05$, Fisher's test). Although four of eight surviving mice (50%) treated with Fe-NTA and vehicle developed adenocarcinomas in two experimental control groups, none of the treated mice developed adenocarcinomas.

Histological findings

In the precancerous stages (Fig. 2), hyperplasia of acidophilic non-ciliated cells (Clara cells) and alveolar duct cells was observed after Fe-NTA treatment. Hyperplasia involving the bronchiolar epithelial and alveolar cells was observed in the alveolar septa with continuous slight occlusion of the blood vessels. Hyperplasia of alveolar cells was observed in the septum with deposits of iron (Fig. 2a). Remarkable deposition of iron was observed in the kidneys. This finding shows that Fe-NTA circulated in the lungs through the blood vessels. Findings were not remarkable in the lungs of mice treated with either propolis or artepillin C. Inflammatory changes were not in interstitial septa or in intra-alveolar spaces.

Pulmonary adenomas arose as adenomas developing from areas with papillary hyperplasia of bronchiolar epithelial cells, and the papillary adenomas exhibited vascular stalks lined with cuboidal or columnar epithelial cells (Fig. 2b, c). In other types of arising adenomas, these consisted of alveolar cells lining the alveolar walls (Fig. 2d).

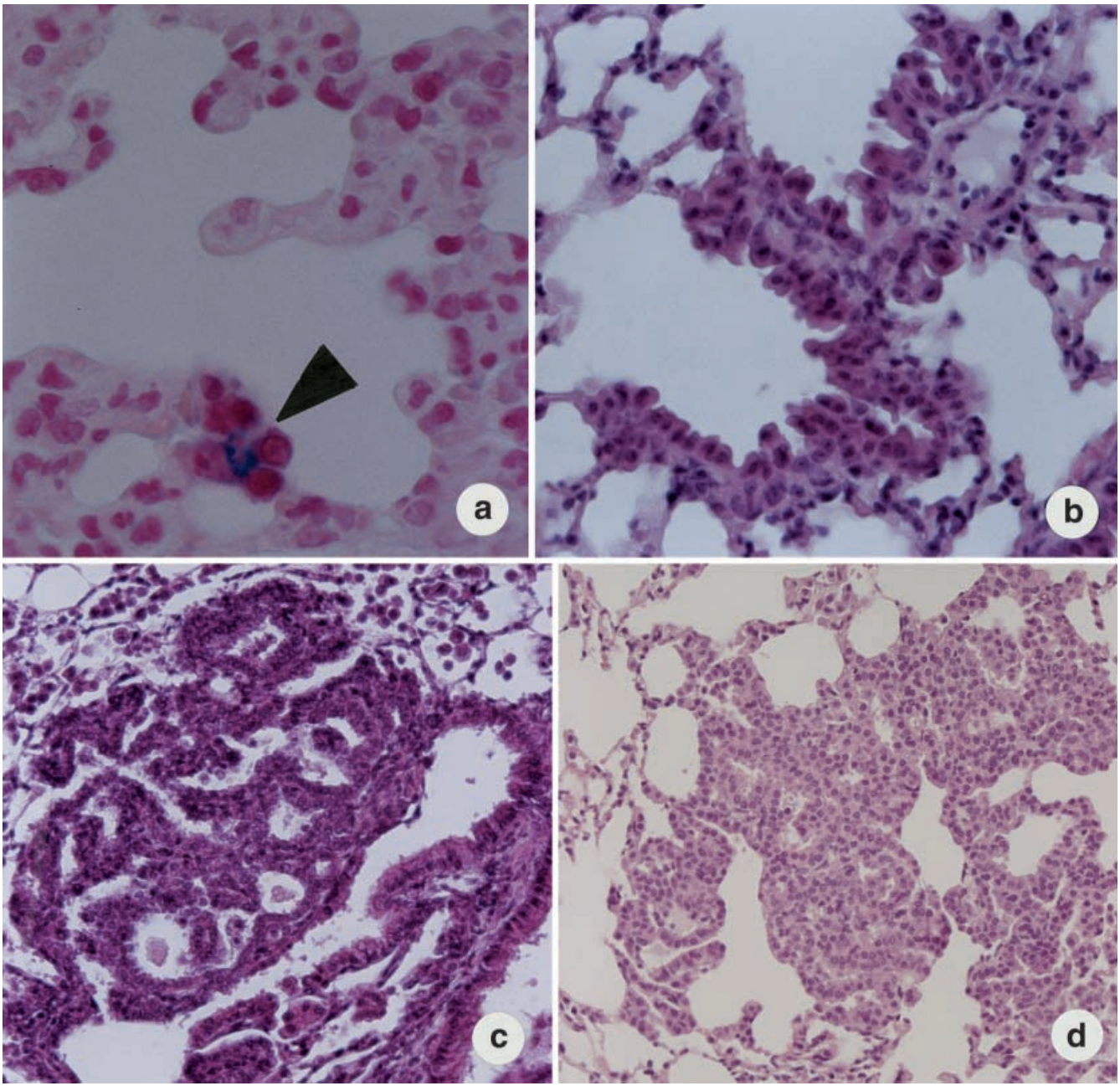

Fig. 2 Hyperplasia and adenoma in mice treated with ferric nitrilotriacetate (Fe-NTA; vehicle control mice, EtOH, or olive oil). **a** Hyperplasia of alveolar cells was noticed in the septum with localization of iron. (*Arrow*, Berlin blue stain, ×200). **b** Proliferation of hyperplastic bronchiolar cells in vehicle control (olive oil) mice. [Hematoxylin and eosin (H&E) stain, ×100]. **c** Papillary adenoma of bronchiolar cells in vehicle control (EtOH) mice. (H&E stain, ×50). **d** Adenoma of alveolar cells in vehicle control (olive oil) mice. (H&E stain, ×50)

Malignant progression from adenomas to adenocarcinomas was observed in the lungs of mice receiving FeNTA only. Pulmonary tumors induced by Fe-NTA were papillary adenocarcinomas derived from bronchiolar epithelial cells (bronchiolar carcinoma, papillary type). The tumor formed a complete trabecular structure with few or multiple layers of cuboidal cells. Cells were irregularly packed on the trabeculae, and scanty spaces were observed between adjacent trabeculae (Fig. 3a). In some lesions, papillary carcinomas were altered with tubular adenocarcinomas. A portion of adenocarcinomas had a glandular arrangement of tumor cells (Fig. 3b), and such lesions of adenocarcinomas were mixed with poorly differentiated adenocarcinomas or large cell carcinomas (Fig. 3c, d). The constituent anaplastic large round cells resembled alveolar cells.

In contrast to what was observed in untreated mice, adenocarcinomas were not observed in mice treated with either propolis or artepillin C. Instead, typical mature adenomas with vascular stalks (bronchiolar pattern,

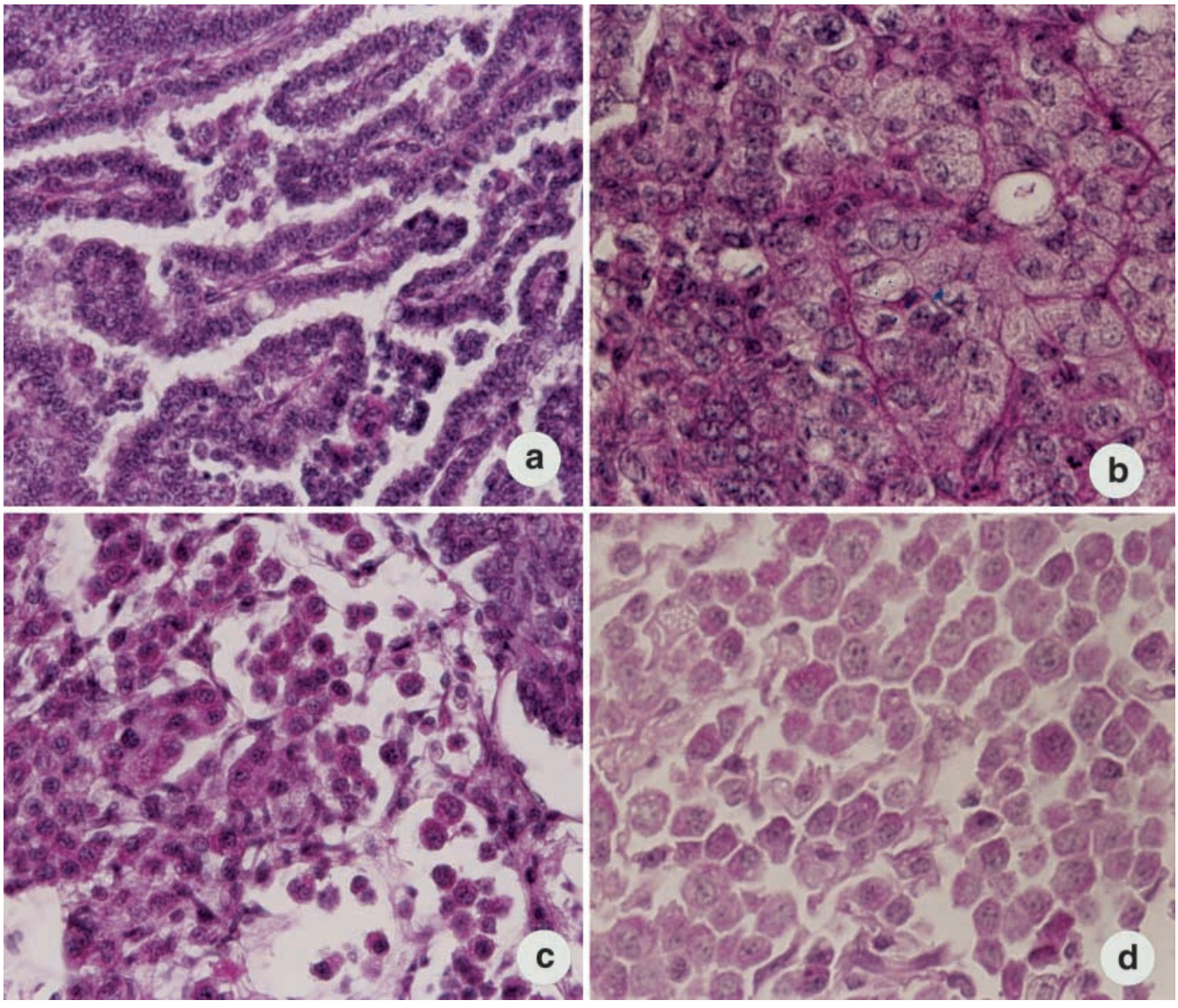

Fig. 3 Histological appearances of pulmonary carcinomas in vehicle control mice (EtOH) induced by repeated injections of ferric nitrilotriacetate (Fe-NTA). **a** Papillary adenocarcinoma of bronchiolar cell origin. [Periodic acid–Schiff (PAS) stain, ×100]. **b** A portion of papillary adenocarcinomas have a glandular arrangement and form tubular adenocarcinomas. (PAS stain, ×124) **c** A lesion of adenocarcinoma was composed of a mixture of poorly differentiated adenocarcinoma and large cell carcinoma cells. (PAS stain, ×100). **d** The constituent anaplastic large round cells resembled alveolar cells. (PAS stain, ×160)

Fig. 4a) and of the alveolar cell type were observed combined with remarkable proliferation of mature alveolar type-II cells (Fig. 4b, c).

PAS staining after Fe-NTA treatment

PAS staining and light microscopic study of the lungs revealed collapse of the blood vessels and swelling of activated alveolar cells (type II) in the thickening septum 5 h after Fe-NTA treatment. Congestion of the septa remained during Fe-NTA treatment and became chronic. During the chronic stage, slight bleeding restricted to the alveolar spaces was observed, but degenerative, necrotic changes and inflammation of the pulmonary tissues were not observed. Reactive, proliferative responses of bronchiolar epithelial or alveolar cells were observed in association with congested blood vessels (Fig. 5).

Histochemical detection of aldehydes (cold Schiff's reaction)

Bronchiolar epithelial and alveolar cells showed diffuse slight positive cold Schiff's reaction 1 h after the FeNTA injection (data not shown). However, bronchiolar epithelial and alveolar cells of mouse lungs treated with propolis or artepillin C were not stained by cold Schiff's reagent, indicating that peroxidation in the lungs was not localized. Treatment with either propolis or artepillin C protected

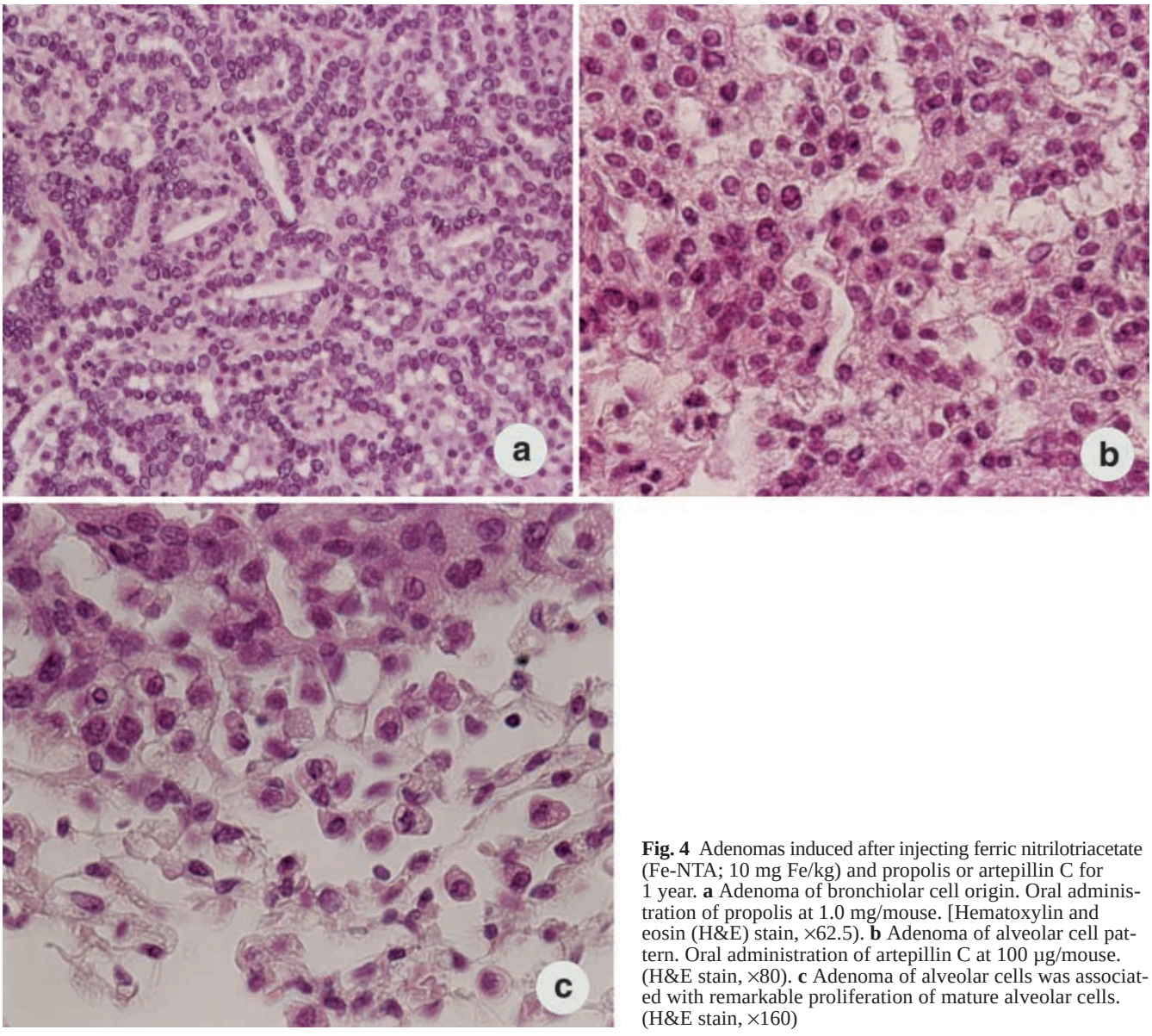

Fig. 4 Adenomas induced after injecting ferric nitrilotriacetate (Fe-NTA; 10 mg Fe/kg) and propolis or artepillin C for 1 year. **a** Adenoma of bronchiolar cell origin. Oral administration of propolis at 1.0 mg/mouse. [Hematoxylin and eosin (H&E) stain, ×62.5). **b** Adenoma of alveolar cell pattern. Oral administration of artepillin C at 100 µg/mouse. (H&E stain, ×80). **c** Adenoma of alveolar cells was associated with remarkable proliferation of mature alveolar cells. (H&E stain, ×160)

Fe-NTA-treated mouse lungs from peroxidation. Although when compared to renal peroxidation, that of the lungs in Fe-NTA-treated mice was weak, Schiff's reactivity was associated with the appearance of activated alveolar cells with large nuclei that were localized to the alveolar septum and congestion of the alveolar septa 5 h after treatment with Fe-NTA.

Detection of 4-HNE-modified protein and 8-OHdG in the lung

4-HNE-modified proteins were detected in the cytoplasm of bronchiolar epithelial and alveolar (type II) cells 1 h after Fe-NTA injection (Fig. 6b, c). Administration of either propolis or artepillin C decreased the formation of these modified proteins and protected the pulmonary tissues from oxidative damage (Fig. 6d, e).

The DNA base-modified product, 8-OHdG, is one of the most generally used markers for the evaluation of oxidation-induced DNA damage [10, 40]. After injecting Fe-NTA, localization and accumulation of 8-OHdG in the nucleus was observed in alveolar cells and in cells of the bronchial epithelium (Fig. 7b). Accumulation of 8-OHdG in the nucleus was not observed after treatment with either propolis or artepillin C (Fig. 7c, d).

PCNA and TTF-1 staining

Cells in the hyperplastic tissues and adenomas of mice injected with Fe-NTA 1 year previously stained positive for PCNA, confirming active proliferation of the cells (Fig. 8a–c). The adenomatous cells also stained strongly positive for TTF-1, indicating that these neoplastic cells

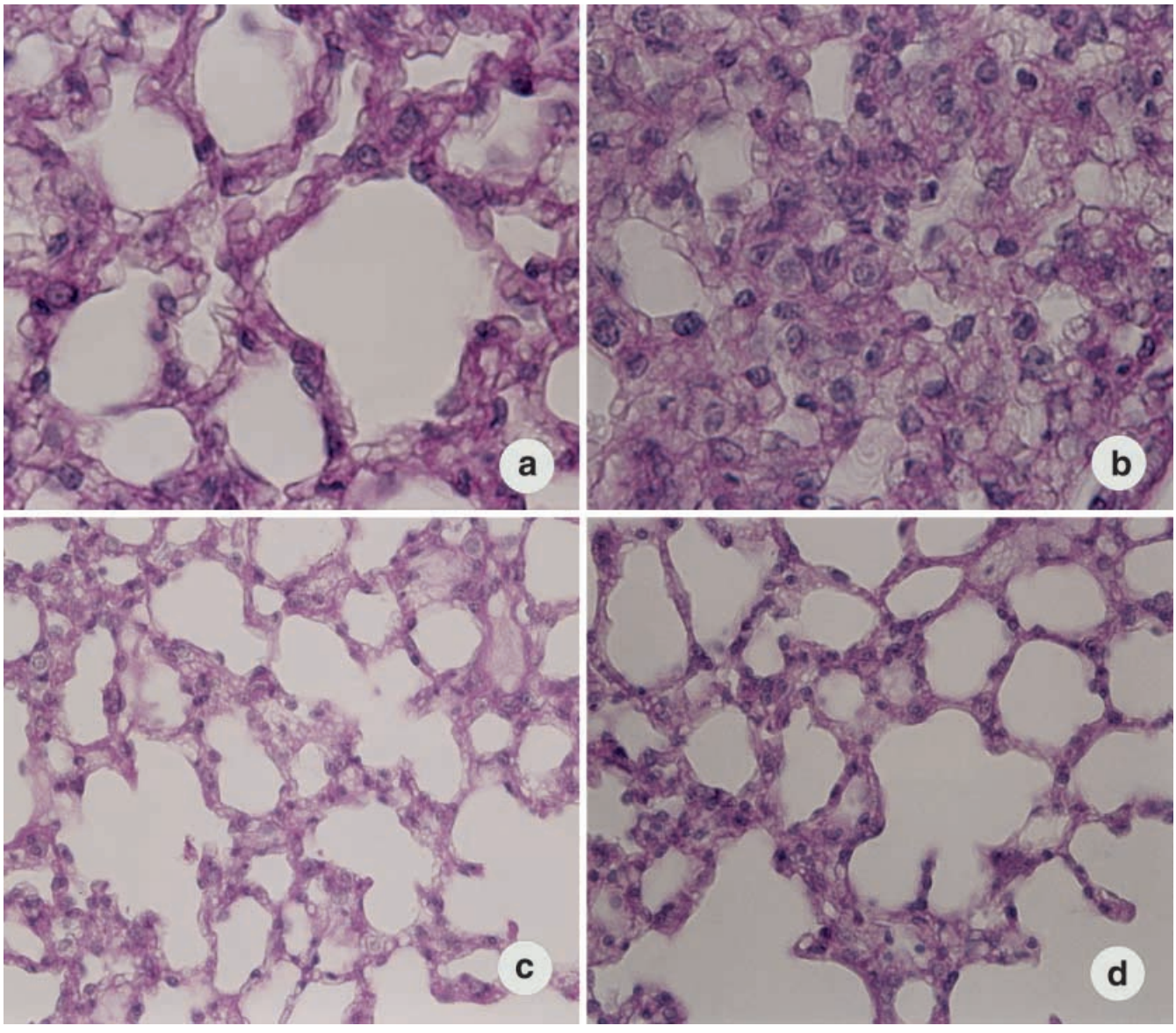

Fig. 5 Histological appearance of the lung 1 h after ferric nitrilotriacetate (Fe-NTA; 7 mg Fe/kg) injection. **a** Untreated normal lung. [Periodic acid–Schiff (PAS) stain, ×200]. **b** Activated alveolar cells (type II) with large nuclei in mice injected with Fe-NTA. (PAS stain, ×200). **c** 1.0 mg/mouse of propolis was administrated orally 2 h before Fe-NTA injection. (PAS stain, x100). **d** 100 µg/mouse of artepillin C was administrated 5 h before Fe-NTA injection. (PAS stain, ×100)

were pulmonary in origin and not metastatic cells originating from renal tumors (Fig. 8d). The nuclei of cells in adenocarcinomas and large cell carcinomas stained strongly positive for TTF-1. Adenocarcinomas were not observed in the mice treated with either propolis or artepillin C, but the occurring adenomas stained positive for PCNA and TTF-1. Infiltrating cells staining positive for CD68 (activated alveolar macrophages) were not observed in adenomas and alveolar type-II tissues in mice receiving Fe-NTA only.

TBARS formation in the lungs

Levels of TBARSs in the lungs were found to increase after injection of Fe-NTA ($P<0.05$), but this was not as strong as that observed in the kidneys, as reported in our previous paper. As reported for the kidneys, the experimental groups receiving propolis or artepillin C showed a slight decrease in levels of TBARSs in their lungs (Fig. 9).

Discussion

In this investigation, we show that the renal carcinogen Fe-NTA can also induce pulmonary adenocarcinomas in surviving mice 1 year after receiving Fe-NTA for 8 weeks. Mice treated with either propolis or artepillin C and surviving the nephrotoxic effects of Fe-NTA developed adenomas in their lungs, but these did not progress to adenocarcinomas. In experiment 2 of our study, 1 year

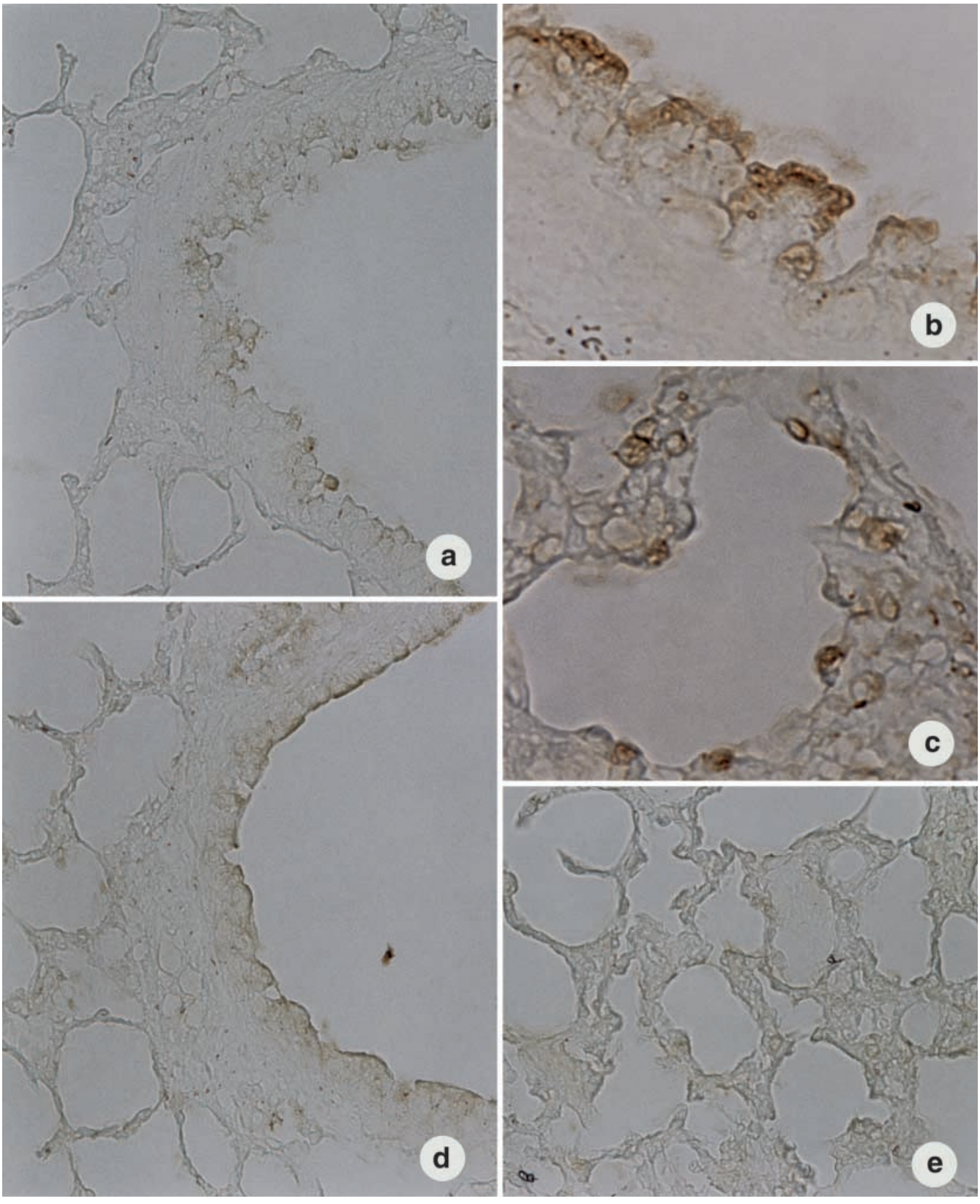

Fig. 6 Immunohistochemical localization of 4-hydroxy-2-nonenal (4-HNE) in the lungs 1 h after ferric nitrilotriacetate (Fe-NTA; 7 mg Fe/kg) injection. **a** Untreated normal lung. Bronchiolar and alveolar cells were negatively stained. (×100). **b** Bronchiolar epithelia of mice treated with Fe-NTA were positively stained. (×200). **c** Alveolar cells of mice treated with Fe-NTA were positively stained. (×200). **d** Bronchiolar cells of mice administered 1.0 mg/mouse of propolis orally. (×100). **e** Alveolar cells of mice administered 100 µg/mouse of artepillin C orally. Inhibition of lipid peroxidation was noticed in bronchiolar and alveolar cells. (×100)

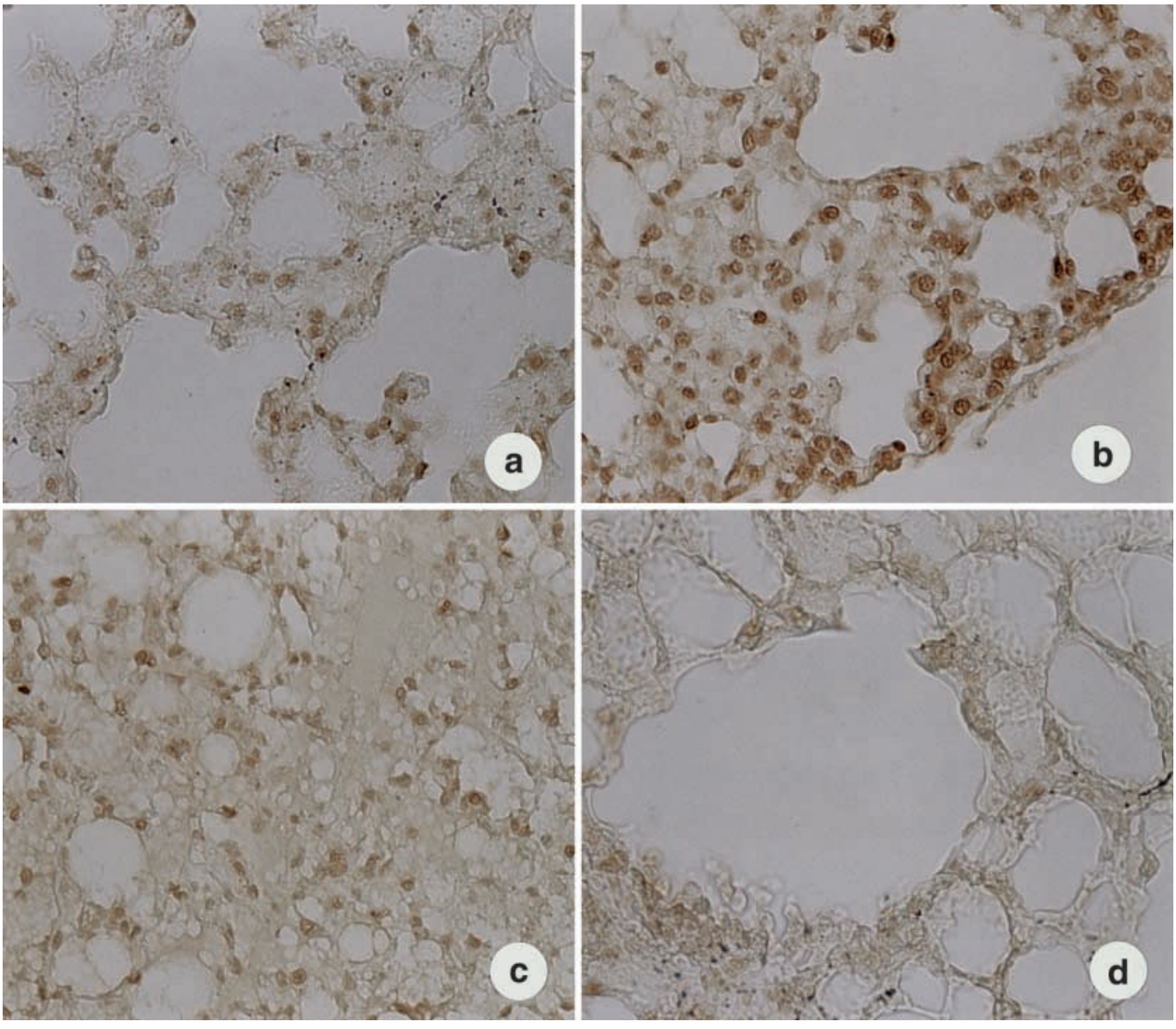

Fig. 7 Immunohistochemical localization of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the lungs 1 h after ferric nitrilotriacetate (Fe-NTA; 7 mg Fe/kg) injection. **a** Untreated normal lung. Alveolar cells were weakly stained positive. (×100). **b** 8-OHdG was localized in alveolar cells. Alveolar cells from mice treated with FeNTA. (×200). **c** Propolis 1.0 mg/mouse was administered 2 h before injecting Fe-NTA. Alveolar cells were weakly stained positive. (×100). **d** 100 µg/mouse of artepillin C was administered 5 h before Fe-NTA injection. 8-OHdG was negatively stained. Inhibition of 8-OHdG localization in alveolar cells treated with propolis and artepillin C (×100)

after injection of Fe-NTA for 8 weeks, a significant 50% of untreated surviving mice developed adenocarcinomas, compared with none in the treated groups. In mice treated with artepillin C, infiltration of activated macrophages (type II) into the adenomas was also observed. Treatment induced a reduction in the intensity of staining for markers of oxidative damage, such as 4-HNE-modified proteins and 8-OHdG in pulmonary tissues. Furthermore, the levels of TBARS in the tissues also decreased in mice treated with either propolis or artepillin C. The neoplastic tissues induced in the lungs of mice by Fe-NTA were stained positive with anti-TTF-1 antibody, confirming that these neoplastic tissues were primary pulmonary tumors and not of renal origin.

Fe-NTA is a non-genotoxic carcinogen, and administration of Fe-NTA induces severe acute renal tubular necrosis with lipid peroxidation in rats and mice [14, 15, 33, 46, 51]. A high incidence of renal adenocarcinomas was observed in rats and mice receiving Fe-NTA induced by oxidative damage. In renal adenocarcinomas, cyst formation precedes cancer development, and the cystic-papillary cancer induced by Fe-NTA is of proximal tubular cell origin [29]. In this study, adenomas of alveolar cells and bronchiolar epithelia progressed into adenocarcinomas associated with high levels of lipid peroxidation in the lungs.

Propolis is a mixture of compounds obtained from beehives. It has various bioactivities, including antibacterial [1, 30], anti-viral [45], and anti-inflammatory

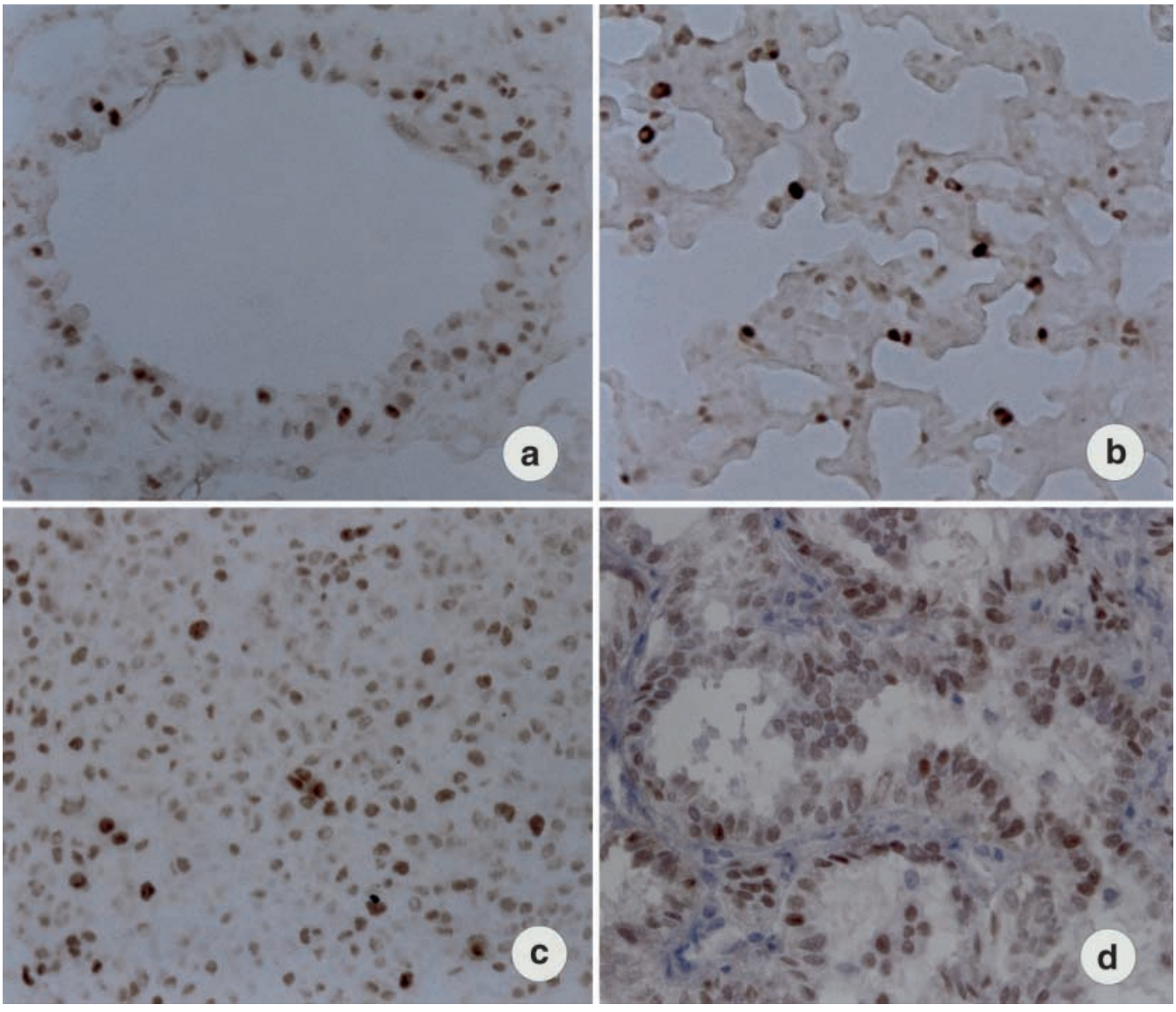

Fig. 8 Immunohistochemistry of proliferating cell nuclear antigen (PCNA) and thyroid transcription factor-1 (TTF-1). **a** Bronchiolar epithelia of control mice treated with ferric nitrilotriacetate (FeNTA) and olive oil. Non-cancerous lesion in surviving mice 1 year after Fe-NTA. (×100). **b** Alveolar cells of control mice treated with Fe-NTA and olive oil. Non-cancerous lesion. (×100). **c** Vehicle control mice (olive oil) were strongly positively stained for PCNA. Lungs of mice injected with Fe-NTA previously were stained positive for PCNA in surviving mice 1 year. (×100). **d** Adenoma of mice induced by treatment with Fe-NTA and artepillin C. TTF-1 was strong positively stained in a part of the adenoma. The adenoma was confirmed to be a primary lung tumor. (×100)

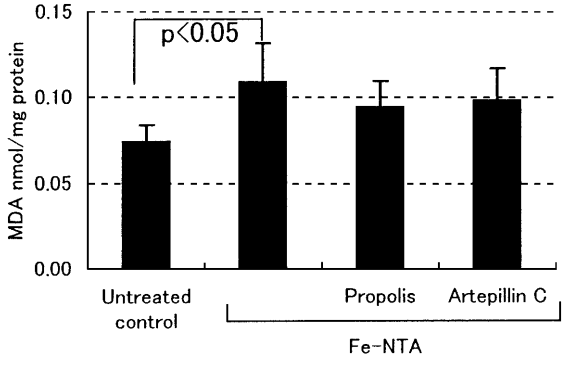

Fig. 9 The effect of propolis and artepillin C on lung thiobarbituric acid-reactive substance levels induced by ferric nitrilotriacetate

[22] effects. We have identified artepillin C as an active substance isolated from propolis and have shown that it exhibited anti-tumor activities and induced cancer cell apoptosis in vivo and in vitro [23, 24]. Additionally, Brazilian propolis and artepillin C possess anti-oxidant effects, and renal carcinogenesis induced by Fe-NTA in mice was prevented. In the same study, using electron spin resonance (ESR), we show that both propolis and artepillin C have anti-oxidative effects [25]. The results confirm that these substances possess scavenging activity for oxyradicals and suggest that they protect mice from

lipid peroxidation in the lungs. Thus, pulmonary damage and carcinogenesis are inhibited by these scavengers. Artepillin C induces apoptosis of malignant cells directly and has inhibitory effects on cancer growth in mice [23, 24]. The low 50% mouse survival following treatment is considered to be due to the severe nephrotoxic effects of Fe-NTA that could not be inhibited by either propolis or artepillin C [50].

In this investigation, pulmonary adenocarcinomas and large cell cancers arising from adenomas consisted of alveolar cells or bronchiolar epithelia. We found that propolis and artepillin C administrated orally prevent the increase in markers of lipid peroxidation, such as TBARSs, 4-HNE, and 8-OHdG in the lungs. Although the levels of TBARSs in the lungs were lower than those reported for the kidneys [25], the levels decreased further after oral administration of either propolis or artepillin C. The renal proximal tubules of male mice showed cold Schiff's reaction positivity 1 h after the FeNTA injection [25], and destruction of the brush border was observed after PAS staining. However, in the lungs, pulmonary alveolar and the bronchiolar epithelium showed only weak Schiff's reaction (data not shown). These histochemical findings confirmed the suppressive effects of propolis and artepillin C on lipid peroxidation in the lungs and confirmed that the administration of either propolis or artepillin C induces local anti-oxidative effects in these tissues. Immunohistochemistry showed that 4-HNE was positive in the cytoplasmic microsomes in bronchiolar epithelia and in part of the alveolar cells after Fe-NTA. However, 4-HNE was absent after treatment with either propolis or artepillin C. 8-OHdG induced by DNA damage due to lipid peroxidaton was detected in the nucleus of alveolar and bronchiolar cells of the sub-pleural regions. It was considered an important finding that 8-OHdG was found in the nucleus of some alveolar cells in untreated control mice but was not found in the bronchiolar epithelium. TBARS was also higher in the lungs than in the kidneys of normal mice. Thus, occasionally a spontaneous adenoma in an untreated mouse lung might arise through lipid peroxidation (Table 1). Congestion of capillary blood vessels in the alveolar septa and proliferation of alveolar type-II cells was observed 1 h after treatment with Fe-NTA. This was inhibited by both propolis and artepillin C.

Morphologically, Clara cells appeared to be pivotal in our model of carcinogenesis. Many investigators have histologically classified cancers on the basis of predominant morphological features as either alveolar or bronchiolar, and the cellular origin of the cancers is identified using electron microscopy [4, 9, 13, 42]. After injection of Fe-NTA, alveolar cancers exhibited an alveolar type-II cell pattern, whereas bronchiolar papillary cancers consisted of non-ciliated epithelial Clara cells of the bronchioles. Clara cells are metabolically the most active of all the cells types comprising the respiratory epithelia. Histochemistry has shown that the highest activity of oxidative enzymes occurs in their cytoplasm. 4-HNE and 8-OHdG induced by lipid peroxidation were noticed in the bronchiolar epithelia. The alveolar type-II epithelia were made up of continuous cords of uniform cuboidal cells lining the alveolar septa, filling the alveolar spaces to give a solid compact appearance. During anaplastic changes of the bronchiolar epithelium, cell mixtures of different phenotypes arise from a common stem cell as proposed by Reznik-Schaller and Reznif [38]. The alveolar cells and Clara cells are rich in chorine-base phospholipid, and these cells are probably injured by lipid peroxidation. The cellular localization of pulmonary oxidase activity has aroused considerable interest, especially because of the increasing incidence of lung disease, including cancers. Evidence for Clara cells as the site of cytochrome-dependent mixed-function oxidase activity in the lung has also been reported [7].

Adenomas arising from both cell types progressed into adenocarcinomas and then to large cell cancers after 8 weeks of repeated injections of Fe-NTA. In contrast, mice receiving oral administrations of either propolis or artepillin C were protected from malignant progression to adenocarcinomas. This study therefore confirms that propolis and artepillin C have chemopreventive effects and inhibit carcinogenesis in histologically distinct tissues, such as the lungs and the kidneys [25], and suggests that these natural products are health preserving substances with prophylactic potential.

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