

Curcumin attenuates oxidative damage in animals treated with a renal carcinogen, ferric nitrilotriacetate (Fe-NTA): implications for cancer prevention

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Received: 12 September 2008 / Accepted: 11 December 2008 / Published online: 23 January 2009
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Abstract Curcumin (diferuloylmethane), a biologically active ingredient derived from rhizome of the plant *Curcuma longa*, has potent anticancer properties as demonstrated in a plethora of human cancer cell lines/animal carcinogenesis model and also acts as a biological response modifier in various disorders. We have reported previously that dietary supplementation of curcumin suppresses renal ornithine decarboxylase (Okazaki et al. *Biochim Biophys Acta* 1740:357–366, 2005) and enhances activities of antioxidant and phase II metabolizing enzymes in mice (Iqbal et al. *Pharmacol Toxicol* 92:33–38, 2003) and also inhibits Fe-NTA-induced oxidative injury of lipids and DNA in vitro (Iqbal et al. *Teratog Carcinog Mutagen* 1:151–160, 2003). This study was designed to examine whether curcumin possess the potential to suppress the oxidative damage caused by kidney-specific carcinogen, Fe-NTA, in animals. In accord with previous report, at 1 h after Fe-NTA treatment (9.0 mg Fe/kg body weight intraperitoneally), a substantial increased formation of 4-hydroxy-2-nonenal (HNE)-modified protein adducts in renal proximal tubules of animals was observed. Likewise, the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and protein reactive carbonyl, an indicator of protein oxidation, were also increased at 1 h after Fe-NTA treatment in the kidneys of animals. The prophylactic feeding of animals with 1.0% curcumin in diet for 4 weeks

completely abolished the formation of (i) HNE-modified protein adducts, (ii) 8-OHdG, and (iii) protein reactive carbonyl in the kidneys of Fe-NTA-treated animals. Taken together, our results suggest that curcumin may afford substantial protection against oxidative damage caused by Fe-NTA, and these protective effects may be mediated via its antioxidant properties. These properties of curcumin strongly suggest that it could be used as a cancer chemopreventive agent.

Keywords Curcumin · Cancer prevention · 4-Hydroxy-2-nonenal · Oxidative stress · 8-Hydroxydeoxyguanosine · Ferric nitrilotriacetate · Protein carbonyl

Introduction

Several lines of evidence indicate that oxidative stress may play an important role in various pathological conditions, including cancer, neurodegeneration, atherosclerosis, diabetes, heart diseases, retinal degeneration, and rheumatoid arthritis, as well as drug-associated toxicity, postischemic reoxygenation injury, and aging [1]. We have developed a model of iron-induced oxidative tissue damage and carcinogenesis using ferric nitrilotriacetate (Fe-NTA), an iron chelate compound in rats and mice [2, 3]. This is a unique model characterized by (i) high incidence of pulmonary metastasis and peritoneal invasion (ii) high incidence of tumor associated mortality, and (iii) possible involvement of reactive oxygen species (ROS) in carcinogenic process [2, 3]. Fe-NTA-induced free radicals cause molecular oxidative damage [4]. The susceptibility of polyunsaturated fatty acids to radical attack results in the destruction of membrane lipids and the production of lipid peroxides and

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their byproducts, such as aldehydes [5]. Malondialdehyde (MDA), and 4-hydroxy-2-alkenals (4-HAD), such as 4-hydroxy-2-nonenal (HNE), are the major end products derived from the breakdown of polyunsaturated fatty acids and related esters [5]. HNE is a highly reactive electrophile that exhibits a variety of cytopathological effects because of its facile reactivity with biological molecules, particularly with proteins [6]. Toyokuni et al. [7, 8] have provided direct evidence for the formation of aberrant proteins, including HNE-modified and MDA-modified proteins, in rat kidney following treatment with Fe-NTA, a consequence of oxidative stress. The involvement of oxidative stress in this model of renal carcinogenesis is also suggested by increased levels of markers of oxidative DNA damage, including the formation of 8-hydroxydeoxyguanosine (8-OHdG) [9–12]. Since 8-OHdG adducts can cause misreading of the DNA sequence during replication, due to G- for -T base substitutions [13], they might result in a significant activation of oncogenes [14]. Accordingly, it seems likely that both of the established products play key roles in Fe-NTA-induced toxicity and carcinogenicity. Although the mechanism of Fe-NTA-induced genotoxicity is not known, it has been suggested that ROS might contribute to its renal carcinogenicity [2–4, 7–14]. Therefore, inhibition of markers of oxidative damage in target organs by antioxidants might be expected to be effective for prevention of radical-mediated carcinogenesis.

Curcumin is a major yellow pigment in turmeric (the ground rhizome of *Curcuma longa* Linn), which is widely used as a spice and coloring agent in several foods, such as curry, mustard, and potato chips, as well as cosmetics and drugs [15, 16]. A wide range of biological and pharmacological activities of curcumin have been investigated [15, 16]. Curcumin is a potent inhibitor of mutagenesis and chemically induced carcinogenesis [17–19]. It possesses many therapeutic properties including anti-inflammatory and anticancer activities [20]. Curcumin is currently attracting strong attention due to its antioxidant potential as well as relatively low toxicity to rodents [15–20]. Curcumin is an inhibitor of neutrophil responses [21] and superoxide generation in macrophages [21]. In previous studies, we have shown that dietary supplementation of curcumin suppresses renal ornithine decarboxylase and enhances the activities of antioxidant and phase II metabolizing enzymes in mice [22, 23] and also inhibits Fe-NTA-induced oxidative injury of lipids and DNA in vitro [24]. These findings support the need for further study. Therefore, the present study was designed to examine whether curcumin possesses the potential to suppress the oxidative damage caused by Fe-NTA. Curcumin was found to suppress Fe-NTA-induced formation of: (i) HNE-modified protein adducts, (ii) 8-OHdG, and (iii) protein reactive carbonyl contents in the kidneys of animals.

Materials and methods

Chemicals

Bovine serum albumin (BSA), curcumin, 2,4-dinitrophenylhydrazine (2,4-DNPH), guanidine-HCl, tris-HCl, ethylenediamine tetraacetic acid (EDTA), sodium bicarbonate, trichloroacetic acid (TCA), ethyl acetate, and nitrilotriacetic acid disodium salt (NTA) were purchased from either Sigma Chemical Company, St Louis, MO, USA, or Aldrich, USA. All other chemicals/reagents were of highest quality available from Wako pure chemical industries Ltd, Osaka, Japan.

Antibodies

Monoclonal antibody, HNEJ-2, specific for HNE-modified protein adducts [25] and N45.1, specific for 8-OHdG [26], respectively, were used for immunohistochemistry and kindly provided by Dr. S. Toyokuni (Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Japan).

Preparation and injection of Fe-NTA solution

A solution of Fe-NTA was prepared by the method of Awai et al. [27] with slight modification. Briefly, ferric nitrate (FeNO_3) and NTA was dissolved in double distilled water. The respective solutions were mixed to achieve a molar ratio of 1:3 of Fe-NTA. The pH was adjusted to 7.4 with sodium bicarbonate with constant stirring. All solutions were prepared fresh immediately before its use. Fe-NTA was injected intraperitoneally into the animals.

Animals and experimental groups

Animal experiments were approved by animal care committee of Okayama University Medical School, care and handling of the animals was in accordance with National Institutes of Health guidelines. Male ddY mice (4–6 weeks old) weighing 20–30 g obtained from Shizuoka Laboratory Animal Centre, Shizuoka, Japan, were used. They were kept in a temperature controlled (25°C) room with alternating 12-h/12-h light/dark cycles, and were allowed to acclimatize for 1 week before study and had free access to standard laboratory chow and water ad libitum. The basic diet without curcumin supplement is referred to as the control diet and that supplemented with 1.0% curcumin is referred to as the curcumin diet.

The animals were divided into four groups consisting of $n = 6$ in each. The animals of groups I & II received control (normal) diet and served as controls, whereas the animals of groups III & IV received a prophylactic

treatment of 1.0% curcumin in diet for 4 weeks. After 4 weeks of dietary supplementation of curcumin, the animals of groups II & III received an intraperitoneal injection of Fe-NTA at a dose of 9.0 mg Fe/kg body weight. All of these animals were killed at 1 h after the treatment with Fe-NTA or saline by cervical decapitation and both kidneys of each animal were removed immediately. One kidney of each animal was fixed in 10% phosphate buffered formalin overnight, and embedded in paraffin, sectioned at 3.5 μm , and mounted on glass slides either for hematoxylin/eosin staining or immunohistochemistry. The other kidney of each animal was homogenized in 0.1 M phosphate buffer, pH 7.4, containing 1.15% KCl, for the preparation of cytosol as described previously [28, 29] and was used for the assay of protein reactive carbonyl contents.

Immunohistochemistry

The immunohistochemical studies were conducted using avidin–biotin complex (ABC) peroxidase method of Hsu et al. [30] as described by Toyokuni et al. [7]. Samples were fixed with 10% phosphate buffered formalin overnight and embedded in paraffin. After deparaffinization and dehydration through a graded xylene/ethanol series, incubation in 3.0% hydrogen peroxide in 10 mM phosphate buffered saline (PBS) was applied for the inhibition of endogenous peroxidase. After these procedures, normal rabbit serum (Dako, Glostrup, Denmark; diluted to 1:10) for the inhibition of non-specific binding of secondary antibody, partially purified mouse monoclonal antibody, HNEJ-2 against HNE-modified protein adducts (10 $\mu\text{g}/\text{ml}$) or N45.1 against 8-OHdG (10 $\mu\text{g}/\text{ml}$), followed by biotinylated rabbit anti-mouse IgG antiserum (Dako; diluted to 1:100) and ABC (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA; diluted to 1:100) were sequentially applied to the sections. Finally, the sections were incubated with liquid 3,3-diaminobenzidine (DAB) for 3 min. Nuclear staining was performed with Harris's hematoxylin solution (controls using preimmune mouse serum and PBS instead of antibody against HNE-modified protein adducts or 8-OHdG showed no or negligible positivity).

Assay of protein reactive carbonyl contents

Protein reactive carbonyl contents in kidney were assayed by the method of Levine et al. [31] as described by Iqbal et al. [28, 29]. An aliquot 0.5 ml (10%, w/v) of renal $105,000\times g$ cytosolic fractions was treated with an equal volume of 2,4-DNPH (0.1%) in 2 N HCl and incubated for 1 h at room temperature. This mixture was treated with 0.5 ml TCA (10%, w/v) and after centrifugation the precipitate was extracted three times with ethanol/ethyl acetate (1/1, v/v). The protein sample was then dissolved

with 2.0 ml of solution containing guanidine hydrochloride (8 M)/EDTA (13 mM)/Tris (133 mM) (pH 7.2) and UV absorbance was measured at 365 nm. The results were expressed as *n* moles of 2,4-DNPH-incorporated/mg protein based on the molar extinction coefficient of $21.0 \text{ mM}^{-1} \text{ cm}^{-1}$. Protein concentration in all samples was determined using a BCA (bicinchonic acid) protein assay kit (Pierce, Rockford, IL).

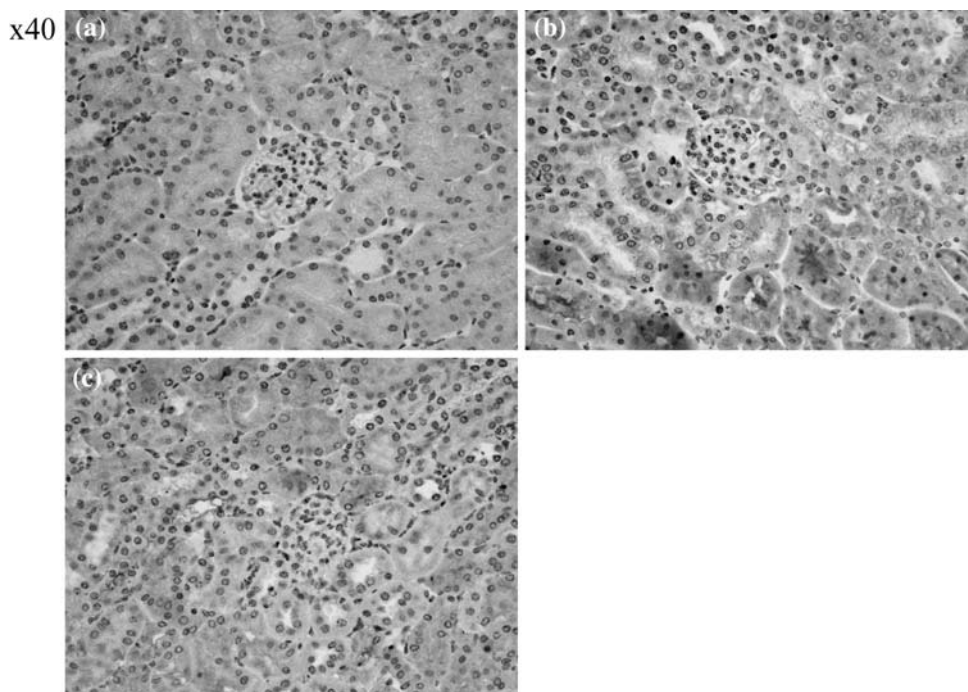
Statistical analysis

Statistical significance was carried out using Student's *t*-test. A *P*-value less than 0.05 was considered as significant difference. All data were expressed as mean \pm SE of six animals.

Results

Since HNE, a major aldehydic product of lipid peroxidation, is believed to be largely responsible for cytopathological effects observed during oxidative stress [5–8], we evaluated the effect of curcumin against Fe-NTA-induced formation of HNE-modified protein adducts in kidney. The results of these investigations, together with the finding that 4-hydroxyalkenals, in particular HNE, are formed during NADPH-Fe-stimulated peroxidation of liver microsomal lipids [32], may help to elucidate the mechanism by which lipid peroxidation causes deleterious effects on cells and cell constituents after Fe-NTA treatment. Immunohistochemical examination revealed that at 1 h after the administration of Fe-NTA there was an increased formation of HNE-modified protein adducts in the form of yellowish brown reaction product in the cytoplasm of the renal proximal tubules (Fig. 1b), presumably resulting from the reaction of HNE with cytoplasmic constituents [32]. These HNE-protein adducts were also detected in the epithelium undergoing necrotic changes in the form of patchy reaction. The tubular lumen contains a small amount of this protein, suggesting that the HNE-modified protein adducts are released in the tubular lumen possibly for its elimination in urine. The HNE-modified protein adducts are primarily detectable in cytoplasm; however, their intranuclear localization could not be ruled out completely. In contrast, prophylactic treatment of animals with 1.0% curcumin in diet almost completely suppressed the Fe-NTA-induced increase formation of HNE-modified protein adducts in kidney (Fig. 1c). In saline-treated control animals, these HNE-proteins were either absent or detected as mild intracytoplasmic reaction products in some renal tubular epithelium (Fig. 1a). Similarly, in curcumin (1.0%) alone-pretreated animals these HNE-proteins were also not detected (data not shown).

Fig. 1 Immunohistochemical appearance of 4-hydroxy-2-nonenal (HNE)-modified protein adducts in the cytoplasm of renal proximal tubule at 1 h after Fe-NTA administration. Dose regimen, treatment protocols, and other details are described in text. **a** Saline-treated control kidney. **b** Fe-NTA-treated kidney (increased formation of HNE-modified protein adducts). **c** Four weeks of curcumin diet (1.0%) pretreatment and Fe-NTA-treated kidney (decreased formation of HNE-modified protein adducts). **a, b, c** $\times 40$. **a–c.** Avidin–biotin complex peroxidase method



Since 8-OHdG, a DNA base modified product, is one of the most commonly used marker for the evaluation of oxidative DNA damage and is thought to be implicated in mutagenesis and carcinogenesis [9–14], we studied the effect of curcumin on Fe-NTA-induced formation of 8-OHdG in kidney. As shown in Fig. 2a, in the renal cortices of saline-treated control animals, faint nuclear staining of renal tubular cells was observed, particularly in the nuclear membranes. Whereas in Fe-NTA-treated animals, intense nuclear staining of renal proximal tubular cells (Fig. 2b), as well as distal tubular cells, was observed. However, 8-OHdG-positive cells were observed to be scattered in the renal proximal tubules even in saline-treated control animals. In contrast to the intense nuclear staining present in Fe-NTA-treated animal's kidney, only slight nuclear staining, almost similar to the staining of saline-treated control animals, was observed in the renal proximal tubules of curcumin and Fe-NTA-treated animals (Fig. 2c). Similarly, a faint nuclear staining was also detected in kidney of curcumin (1.0%) alone-pretreated animals (data not shown).

Because studies have shown that oxidation of some amino acid residues of proteins such as lysine, arginine, and proline leads to the formation of carbonyl derivatives that affects the nature and function of proteins [33]. The presence of carbonyl groups has become a widely accepted measure of oxidative damage of proteins under the conditions of oxidative stress, which react with 2,4-DNPH to form stable hydrazone derivatives [34]. Therefore, we analyzed the effect of curcumin on Fe-NTA-induced

formation of protein reactive carbonyl contents in kidney as a measure of oxidative damage. As shown in Table 1, at 1 h after Fe-NTA treatment, a 1.2-fold increase in the level of protein reactive carbonyl contents in kidney comparison with the saline-treated control was observed. Curcumin pretreatment was thus able to almost completely prevent Fe-NTA-induced oxidation of protein, as monitored by measuring the formation of protein reactive carbonyl contents. Curcumin alone treatment (1.0% diet) was without any effect on the formation of protein reactive carbonyl contents in kidney (data not shown).

Discussion

Recently, there has been an increasing interest in protective function of dietary antioxidants, which are candidates for cancer chemoprevention and for extending lifespan [35]. Several antioxidants, such as vitamin E, vitamin C, β -carotene, uric acid, ubiquinol, and flavonoids, have been found to play important roles in the non-enzymatic protection against oxidative stress [35–38]. However, it has been pointed out that one component in these antioxidants is not enough to prevent carcinogenesis. Therefore, a small number of the components of food are anticipated to have effects on the prevention of carcinogenesis. The efficacy of these chemopreventive agents has been related to their antioxidant potential of reducing/or inhibiting free radical mediated damage to DNA, lipids, and proteins [35–38]. In addition, anticarcinogenic effects of these agents are also

Fig. 2 Immunohistochemical appearance of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the nuclei of renal proximal tubule at 1 h after Fe-NTA administration. Dose regimen, treatment protocols, and other details are described in text. **a** Saline-treated control kidney. **b** Fe-NTA-treated kidney (increased formation of 8-OHdG). **c** Four weeks of curcumin diet (1.0%) pretreatment and Fe-NTA-treated kidney (decreased formation of 8-OHdG). **a, b, c** $\times 40$. **a–c** Avidin–biotin complex peroxidase method

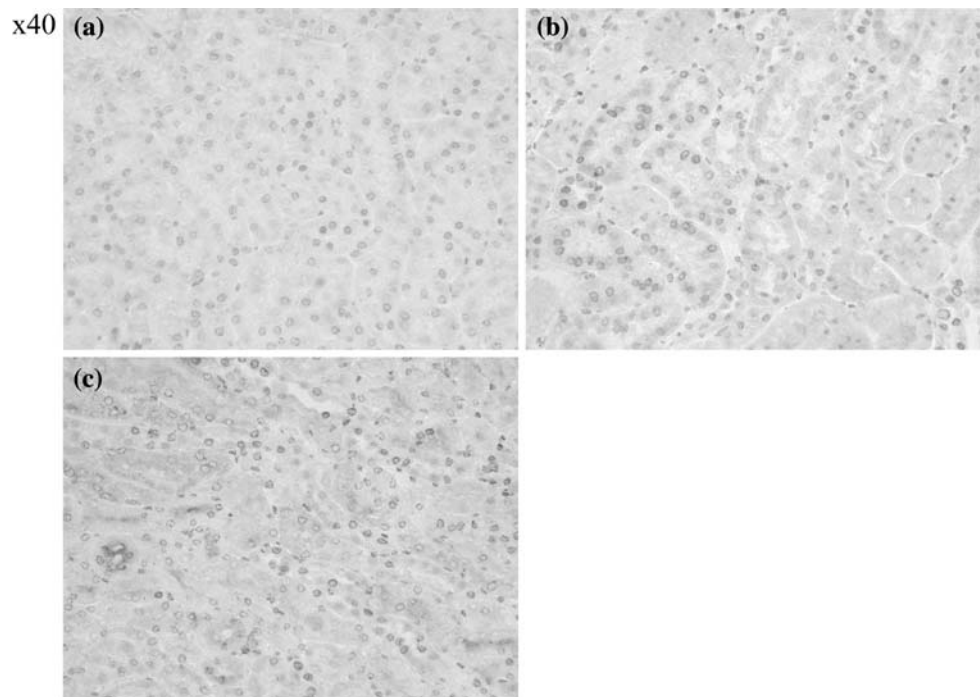


Table 1 Inhibitory effect of curcumin on Fe-NTA-induced formation of protein reactive carbonyl contents in kidney

Experimental groups	Protein reactive carbonyl contents (nmoles of 2,4-DNPH incorporated/mg protein)	Percentage of control
Saline alone	0.38 \pm 0.01	100
Fe-NTA alone	0.48 \pm 0.05*	126
Curcumin (1.0%) + Fe-NTA	0.39 \pm 0.01**	102

Each value represents mean \pm SE of six animals

Dose regimen, treatment protocols, and other details are described in text

* $P < 0.05$ versus saline treatment control group; ** $P < 0.05$ versus Fe-NTA treatment group

reported to be related to their potential of decreasing oxidative stress [35–38] and/or inducing antioxidant and phase II metabolizing enzymes [23].

Oxidative damage to biomolecules has been postulated to be involved in several chronic diseases [39]. Fe-NTA is known as a complete renal carcinogen as well as renal and hepatic tumor promoter [2, 3, 40], acting by inducing ROS generation in the tissues. It was also shown that Fe-NTA down-regulates hepatic and renal quinone reductase activity and produces an increase in protein reactive carbonyl contents [28, 29]. Toyokuni et al. [11, 12] have reported that all four DNA bases are modified in the renal chromatin of rats within 24 h of Fe-NTA treatment. These lesions are typical products of hydroxyl radical reactions. The ability

of Fe-NTA to promote DNA and membrane damage has been postulated as a critical mechanism in renal carcinogenesis associated with the intraperitoneal injection of this complex [7–12, 29]. Several antioxidants have been reported to show protective effects against Fe-NTA-induced toxicity and carcinogenesis [24, 41–45]. In this study, we investigated the antioxidant effects of dietary curcumin, on renal oxidative damage incurred by Fe-NTA.

Because HNE is considered to be the most reliable index of free radical-induced lipid peroxidation and exhibits variety of cytopathological effects such as enzyme inhibition, inhibition of DNA and RNA synthesis, inhibition of protein synthesis, and induction of heat shock proteins [6]. It is highly cytotoxic to many types of cells such as hepatocytes, mammalian fibroblast, and Ehrlich ascites tumor cells [6]. Furthermore, this aldehyde has genotoxic and mutagenic effect as well as inhibitory effect on cell proliferation [6]. It has also been proposed that HNE exerts these effects because of its facile reactivity with biological molecules, particularly with protein via its reactive aldehyde group, is a key factor in the toxicity and carcinogenicity of Fe-NTA [7, 8, 29]. Thus, we were prompted to study whether dietary curcumin is able to block the increase formation of HNE—modified protein adducts in kidney of Fe-NTA-treated animals. Our data demonstrated that prophylactic treatment of curcumin to animals can efficiently attenuate this increase, suggesting the preventive potential of curcumin and indicating that antioxidant property may be responsible for the biological effects of curcumin.

In our next experiment, we assessed the effect of dietary curcumin on Fe-NTA-mediated increase in the formation of 8-OHdG in kidney. A great amount of evidence points to the role of oxidative DNA damage in carcinogenesis [9–14]. The most ubiquitous oxidative DNA base modification is 8-OHdG, and the increase in urinary excretion of 8-OHdG reflects the oxidative DNA damage in vivo [9, 46]. It has been established that Fe-NTA administration results in enhanced formation of 8-OHdG and high incidence of renal carcinoma in rats and mice [2, 3, 9–12, 46]. Lipid peroxidation has been shown to promote the formation of 8-OHdG by production of HNE-modified protein adducts [7, 8]. In accordance with the previous reports [7, 47], we have demonstrated that the levels of 8-OHdG in renal DNA change nearly in parallel with urinary 8-OHdG after Fe-NTA treatment [48], and curcumin suppresses such an increase in 8-OHdG formations. These findings are consistent with our previous in vitro report [24] and other earlier observations that several antioxidants such as 2-mercaptoethanol and *N*-acetylcysteine prevent Fe-NTA-mediated DNA damage [47]. Considering the number of reports demonstrating a close relation between 8-OHdG formation and carcinogenicity [7–14], it seems likely that 8-OHdG formations might participate in Fe-NTA-induced renal carcinogenesis [49]. In this respect, our data revealing that curcumin administration can protect against such an increase in 8-OHdG formation, suggest that curcumin might be effective against Fe-NTA-mediated carcinogenicity by acting as an antioxidant. Though the mechanism of 8-OHdG formation due to cellular oxidation is unclear, the present data are of interest in highlighting a possible link between lipid peroxidation and oxidative DNA damage. HNE also reacts with deoxyguanosine, resulting in the formation of exocyclic adducts [50]. In the presence of *t*-butylhydroperoxide, HNE is readily epoxidized to yield 2,3-epoxy-4-hydroxynonanal, which is more mutagenic and tumorigenic than the parent aldehyde [51].

Our next experiments were directed toward further evaluating whether the preventive effect of curcumin for Fe-NTA-caused damage is mediated via its antioxidant potential. Our data clearly demonstrated that prophylactic treatment of animals with curcumin inhibited Fe-NTA-induced protein oxidation as monitored by measuring protein reactive carbonyl contents in kidney. The method of protein 2,4-DNPH post labeling originally introduced by Levine et al. [31], for isolated proteins has been demonstrated in this study to be useful monitor of Fe-NTA-mediated oxidative damage. Based on the facts [52] that free radicals convert amino acid side chains to carbonyl moieties in vitro, it was believed that method was specific for oxidized proteins. However, studies [53–56] have shown that the attachment of histidine, cysteine, and lysine residues of the proteins to the α,β -double bond of HNE also

generates protein carbonyl groups detected by reaction with 2,4-DNPH and by tritium labeling via reduction of carbonyl groups with tritiated sodium borohydride. The origin of carbonyl group in renal proteins of Fe-NTA-treated animals is still invalid; however, there is no doubt that protein carbonyl measurement using the 2,4-DNPH post-labeling method is useful for monitoring free radical action in vivo and provides indirect evidence that free radical-induced covalent modification of renal proteins occurs in this carcinogenesis. Thus our data clearly demonstrated that curcumin treatment inhibits Fe-NTA-induced protein oxidation as monitored by measuring the formation of protein reactive carbonyl contents in kidney provides further strong clear indication that this agent imparts its preventive action, at least in part, via its antioxidant property.

Conclusion

In brief, our study clearly demonstrated that dietary curcumin afford strong protection against oxidative damage caused by Fe-NTA exposure and these protective effects may be mediated via its strong antioxidant properties. However, because the markers studied here are regarded as early markers of renal carcinogenesis, we suggest that curcumin may be developed as a potent cancer chemopreventive agent against renal carcinogenesis and other adverse effects of Fe-NTA exposure. The results described herein may contribute toward a better understanding of the biological mechanisms associated with health protection by curcumin.

Acknowledgments Authors are thankful to Japan Society for the Promotion of Science (JSPS) for providing grant-in-aid for scientific research to support these studies. Authors are also thankful to Dr. S. Toyokuni (Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Japan) for providing monoclonal antibody, HNEJ-2, specific for HNE-modified protein adducts and N45.1, specific for 8-OHdG, respectively. Authors are also thankful to Dr. Nur Asikin (School of Medicine, University Malaysia Sabah) for reading the manuscript and valuable suggestions.

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