Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 827 (2005) 119–126
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Susceptibility of actin to modification by 4-hydroxy-2-nonenal

Munetaka Ozeki [a], Aya Miyagawa-Hayashino [a], Shinya Akatsuka [a], Tomoyuki Shirase [a], Wen-hua Lee [a], Koji Uchida [b], Shinya Toyokuni [a,*]

[a] *Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan*
[b] *Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*

Received 8 December 2004; accepted 10 February 2005
Available online 10 May 2005

Abstract

4-Hydroxy-2-nonenal (HNE), a major lipid peroxidation product, reacts with histidine, lysine or cysteine residues of proteins to form hemiacetal Michael adducts and thus interferes with the functions of the proteins. Here we undertook to identify HNE-modified proteins in the target organ of a ferric nitrilotriacetate (Fe-NTA)-induced renal carcinogenesis model with histidine-specific HNEJ-2 antibody. Immunoaffinity column separation and sequencing identified one of the major modified proteins as actin. To further explore the characteristics of actin as an HNE acceptor, we produced four novel monoclonal antibodies against HNE-modified keyhole limpet hemocyanin. All these antibodies (HNEJ-1, 3–5) recognized histidine adducts, but were different from HNEJ-2 in recognizing lysine and cysteine adducts to some extent. Actin, albumin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), metallothionein and superoxide dismutase were treated in vitro with HNE and evaluated with these antibodies. The results revealed that actin was most sensitive to HNE modification and metallothionein most resistant. Furthermore, the residue-specificity of GAPDH was in accord with that shown by our recent mass spectrometry data. Immunohistochemistry with the antibodies revealed cytoplasmic staining with or without nuclear staining in the renal proximal tubules after Fe-NTA administration. The results suggest that actin is a major target protein for HNE modification in vivo, and that our monoclonal antibodies are useful for evaluating the HNE adducts produced.

Keywords: 4-Hydroxy-2-nonenal; Monoclonal antibody; Oxidative stress; Protein modification; Kidney; Iron; Carcinogenesis; Michael adducts

1. Introduction

Several lines of evidence indicate that oxidative modification of proteins and the subsequent accumulation of the modified proteins occur in cells and tissues during aging, carcinogenesis and various pathological conditions, including atherosclerosis, autoimmune diseases and neurodegenerative diseases [1,2]. The important chemicals that give rise to the covalent modification of proteins may include reactive aldehydes, such as keto aldehydes, 2-alkenals and 4-hydroxy-2-alkenals [3,4]. These reactive aldehydes are considered to be important mediators of cellular damage due to their ability to covalently modify biomolecules in ways that may disrupt important molecular structures, cause genetic alterations or activate certain signal pathways [4].

4-Hydroxy-2-nonenal (HNE), one of the reactive aldehydes, is a major product of lipid peroxidation [4–6] and is believed to be largely responsible for the cytopathologic effects observed during oxidative stress. HNE exerts these effects because of its strong reactivity with biomolecules, especially cysteine, histidine and lysine residues of proteins, producing cyclic hemiacetal Michael adducts [4].

Administration of an iron chelate, ferric nitrilotriacetate (Fe-NTA), provides an intriguing model of iron-mediated oxidative stress-induced carcinogenesis: intraperitoneal administration of this chemical causes renal proximal tubular damage as a consequence of the Fenton reaction that ultimately leads to up to 90% incidence of renal cell carcinoma in rodents [7–9]. This model is characterized by (1) high incidence of pulmonary metastasis and peritoneal invasion, (2) an

* Corresponding author. Tel.: +81 75 753 4423; fax: +81 75 753 4432.
E-mail address: toyokuni@path1.med.kyoto-u.ac.jp (S. Toyokuni).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.02.025

increase in molecules covalently modified by reactive oxygen species [10] in the kidney during carcinogenesis, and (3) reduction of not only acute renal tubular damage but also tumor incidence [11] by pretreatment with a lipophilic antioxidant, α-tocopherol. We previously reported an increase in a variety of oxidatively modified molecules such as 8-oxoguanine [12], thymine-tyrosine cross-links [13], saturated and unsaturated mutagenic aldehydes including HNE and malondialdehyde (MDA), and HNE- and MDA-modified proteins [6,14,15] in the kidney after Fe-NTA administration. Recently, we identified the $p15^{INK4B}$ and $p16^{INK4A}$ tumor suppressor genes [16,17] and the *annexin 2* gene [18] as target genes in this model.

In the present study, we attempted to identify major HNE-modified proteins in the kidneys of rats after a single administration of Fe-NTA, and identified actin as one of them. Furthermore, we evaluated the characteristics of actin as an HNE acceptor by comparing its immunoreactivity with those of selected proteins using five different monoclonal antibodies (mAbs) produced against HNE-modified keyhole limpet hemocyanin (KLH).

2. Experimental

2.1. Materials

trans-4-Hydroxy-2-nonenal was prepared as previously described [19] by acid treatment (1 mM HCl) of HNE diethylacetal, which was synthesized according to the procedure of De Montarby et al. [20]. The concentration of the HNE stock solution was determined by measurement of UV absorbance at 224 nm [21]. Ferric nitrate enneahydrate was from Wako (Osaka, Japan); nitrilotriacetic acid (NTA) disodium salt was from Nacalai Tesque Inc. (Kyoto, Japan). *N*α-acetyl-L-histidine, *N*α-acetyl-L-lysine, glutathione, bovine muscle actin, rabbit metallothionein and bovine superoxide dismutase (SOD) were from Sigma (St. Louis, MO). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Calbiochem (La Jolla, CA) and bovine serum albumin (BSA), protease-free, was from Nacalai Tesque. All the chemicals used were of analytical quality and deionized water was used throughout. Fe-NTA solution was prepared immediately before injection as previously described [14]. Briefly, ferric nitrate enneahydrate and NTA disodium salt were each dissolved in deionized water to form 300 and 600 mM solutions, respectively. They were mixed at a volume ratio of 1:2 (molar ratio, 1:4) with magnetic stirring at room temperature (RT). The pH was adjusted with sodium carbonate to 7.4.

2.2. Modification of proteins with HNE

Each protein (1 mg/ml) was incubated with 1 or 2 mM HNE for 1 h at 37 °C in 0.1 M phosphate buffer, pH 7.4 as described [22], and dialyzed against phosphate buffer to remove free HNE.

2.3. Animal experiments

The acute study of a single intraperitoneal injection of Fe-NTA (15 mg iron/kg; 0, 3, 6, 12, 24, 48 and 96 h) was carried out as previously described using 6 week-old specific pathogen-free male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan). This experiment was approved by the committee for animal experiments in the Graduate School of Medicine, Kyoto University. The animals were killed at the assigned time after Fe-NTA administration. Both of the kidneys were immediately excised and stored frozen at −80 °C until use. A small portion of the kidney was fixed with Bouin's solution [23] overnight for histological examination with hematoxylin and eosin staining or immunohistochemistry of paraffin-embedded specimens [14].

2.4. Antibodies

MAbs HNEJ1-5 were produced and purified as previously described [19]. Briefly, the HNE-modified KLH (HNE-KLH) immunogen was prepared by reacting KLH with HNE as described [22]. HNE-modified BSA (HNE-BSA) was prepared by incubating 1 mg/ml of BSA with 2 mM HNE at 37 °C for 1 h. HNE-KLH (100 μg/100 μl) was emulsified in an equal volume of Freund's complete adjuvant (Difco, Detroit, MI) and injected intraperitoneally, subcutaneously and intramuscularly (1/2, 1/4 and 1/4 volume, respectively) to female BALB/c mice (7 weeks of age; Shizuoka Laboratory Center, Shizuoka, Japan). After 4 and 8 weeks, booster injections were done. The antibody level in serum was monitored by Western blotting and enzyme-linked immunosorbent assay (ELISA) using BSA and HNE-BSA as paired antigens as described below. Fusion was performed by the use of 8-azaguanine-resistant murine myeloma P3X63-Ag8.653 cells at the ratio of 5:1 as described [24]. The culture medium was RPMI-1640 in the presence of 10% fetal calf serum, 0.03% (w/v) L-glutamine, 0.0015% (w/v) 8-azaguanine and 2.5×10^{-7} M 2-mercaptoethanol. Screening was done with ELISA using BSA and HNE-BSA as paired antigens as described below. MAbs were purified by ammonium sulfate precipitation from ascites of nude mice (BALB/c, nu/nu, female, 8 weeks of age) after intraperitoneal injection of 10^7 hybridoma cells as described [24]. Immunoglobulin subclass was determined using a kit from Amersham Pharmacia Biotech.

Anti-pan-actin mAb (clone MAB1501) was purchased from Chemicon International Inc. (Temecula, CA). Horseradish peroxidase-conjugated rabbit antibody against mouse IgG was from Amersham Pharmacia Biotech (Piscataway, NJ) and biotin-labeled rabbit antibody against mouse IgG was from Dako (Kyoto, Japan).

2.5. ELISA

For monitoring antibody response and cloning hybridomas, the wells of 96-well microtiter plates were filled with

100 μl of either 1 mg/ml BSA or HNE-BSA, and incubated at 4 °C overnight. After three washes with 10 mM phosphate-buffered saline, pH 7.4 (PBS) containing 1% BSA, 0.5% Tween 20, each well was filled with 200 μl of PBS/BSA to block non-specific binding for 1 h at room temperature (RT). After three washes, 150 μl of serum (diluted 1:100) or culture supernatant (diluted 1:3) was added to each well and the plates were incubated for 1 h at RT. Pre-immune BALB/c mouse serum or isotype-matched irrelevant commercial mAb (Dako) was used as a negative control. After three washes, 200 μl of peroxidase-conjugated anti-mouse IgG antiserum (1:1000) was added and the plates were incubated for an additional 1 h at RT. After washing, 100 μl of 0.05 M citrate buffer (pH 5.0) containing 0.4 mg/ml of *o*-phenylenediamine (Wako, Osaka) and 0.015% H_2O_2 was added and the plates were incubated for several minutes at RT. The reaction was terminated by the addition of 2 M sulfuric acid and was quantitated by measuring absorbance at 490 nm with a microtiter plate reader (M-Emax; Molecular Devices, Menlo Park, CA). For studies of HNE specificity and competition, HNE-modified GAPDH and the haptens (HNE-*N*-acetylhistidine, HNE-*N*-acetyllysine and HNE-glutathione) were prepared and a competitive ELISA was carried out as previously described [22].

2.6. Purification of HNE-modified proteins from rat kidney

Eight rats were killed 3 h after administration of 15 mg iron/kg of Fe-NTA, and the kidneys were excised immediately. After homogenization of the kidney sample with 9 volumes of 10 mM Tris–HCl buffer (pH 7.5) containing 0.25 M sucrose, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride, the cytosolic soluble fraction was obtained by ultracentrifugation (105,000 × *g*, 60 min, 4 °C). The supernatant was recovered (∼30 ml) and passed through a Sepharose 4B column (Ø11 mm × 20 mm) to avoid non-specific binding. The HNEJ-2 Sepharose 4B column (Ø17 mm × 65 mm) was prepared by coupling HNEJ-2 to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to the procedures recommended by the manufacturer and the excess remaining groups were blocked by incubation with 0.1 M Tris–HCl buffer, pH 8.0, at 4 °C overninght. Then, an aliquot of the sample was loaded onto the HNEJ-2 Sepharose 4B column according to the procedures recommended by the manufacturer. After the column was washed with 10 mM phophate-buffered saline and 1 M NaCl, bound proteins were eluted with 0.2 M Glycine–HCl buffer (pH 2.5) and analyzed by 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.7. Immunoprecipitation and Western blot analysis

Tissue lysates were prepared by homogenization in RIPA buffer (20 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) in the presence of protease inhibitors (Complete Mini®, Roche, Tokyo, Japan), and the supernatants collected after centrifugation at 15,000 × *g* for 15 min were used. The protein concentration was determined with BCA protein reagents (Pierce, Rockford, IL). Immunoprecipitation was performed as described [18]. Briefly, 10 μl of HNEJ-2 mAb (5 mg/ml) was added to 400 μg of lysate in a 100 μl volume, and incubated on ice for 3 h, followed by addition of protein A Sepharose CL-4B (Amersham Pharmacia Biotech) and incubation on ice for 1 h. After washing with 100 mM HEPES buffer (pH 8.0), the Sepharose beads were separated by centrifugation, and boiled with 50 μl of Laemmli sample buffer containing 2-mercaptoethanol for 3 min. Twenty microliters of the supernatant was used for each lane. Western blotting was carried out after 12.5% SDS–PAGE under reducing conditions (4 μg of protein in each lane) and semi-dry blotting on polyvinylidene difluoride membranes (Immobilon, Millipore, Tokyo, Japan) as described [25]. Briefly, after the membranes were blocked with Blockace (Yukijirushi Co. Ltd., Sapporo, Japan), they were incubated sequentially with primary antibody (HNEJ 1–5, 20 μg/ml; anti-actin monoclonal antibody, diluted 1:2000) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako Japan Co. Ltd., Kyoto, Japan; diluted 1:2000) after washing with 0.1% Tween-20, 100 mM Tris-buffered saline (TTBS, pH 7.4). ECL detection reagents (Amersham Pharmacia Biotech, Tokyo, Japan) were finally used according to the manufacturer's instructions for visualization of the signals on film. The exposure time of the membrane to the film was 1 min.

2.8. Immunohistochemical analysis

The animals were killed 3 h after administration of 15 mg iron/kg of Fe-NTA, and a portion of the kidney was fixed with Bouin's solution. The avidin–biotin complex method with peroxidase was used for immunohistochemistry as described [26]. Serial sections were prepared to enable better comparison of each immunostaining. Briefly, after deparaffinization, the specimens were incubated with 0.3% H_2O_2 in methanol for the inhibition of endogenous peroxidase. Then, normal rabbit serum (diluted 1:75; Dako) for inhibition of non-specific binding of the secondary antibody, mAbs against HNE-modified proteins (HNEJ-1–5; 50 μg/ml), biotin-labeled rabbit anti-mouse IgG serum (diluted 1:300; Dako) and avidin-biotin complex (diluted 1:100; Vector Laboratories, Burlingame, CA) were sequentially used. 3,3′-Diaminobenzidine solution (Dako Liquid DAB, K3466) was used for color development. Three registered pathologists (AMH, TS and ST) independently evaluated the immunostaining without the information of antibody used, and the results these three agreed was used. Procedures using nonimmune mouse IgG_1 or 10 mM PBS instead of HNEJ-1–5 antibody showed no positivity.

2.9. Amino acid sequencing

HNE-modified proteins recovered from the affinity column were separated by SDS–PAGE and portions of the target bands were collected. They were sequenced by a commercial amino acid sequencing service (AproScience, Tokushima, Japan).

3. Results

3.1. Identification of a major HNE-modified protein in the kidney after Fe-NTA administration

SDS–PAGE analysis of the sample after HNEJ-2 column separation revealed two major bands of ∼33 and ∼46 kilodaltons (kDa) (Fig. 1A). The band of ∼46 kDa was subjected to amino acid sequencing. The obtained partial sequence was DLYANTVLSG, which was identified as an actin fragment using NCBI database analysis (http://www.ncbi.nlm.nih.gov/BLAST). Immunoprecipitation and Western blot analysis confirmed the results (Fig. 1B). Analysis of actin in the kidney 3 h after Fe-NTA administration revealed bands with smaller molecular weight as well, suggesting fragmentation, and at least two fragments showed HNE modification (Fig. 1B). Western blot analysis with HNEJ-2 or actin antibodies after SDS–PAGE revealed a minor band with a lower molecular weight of ∼41 kDa. This minor band was detectable in the control sample and was significantly increased between 3 and 24 h after Fe-NTA administration, and then returned to the normal level at 48 h.

Table 1
Affinity of monoclonal antibodies to each 4-hydroxy-2-nonenal-induced Michael adduct

Clone	Histidine-adduct	Lysine-adduct	Cysteine-adduct
HNEJ-1	High	High	High
HNEJ-2[a]	High	Low	Low
HNEJ-3	High	Moderate	Moderate
HNEJ-4	High	Moderate	Moderate
HNEJ-5	High	High	Moderate

[a] See reference [19].

3.2. Characterization of four monoclonal antibodies against HNE-modified proteins

In order to explore the characteristics of actin as an HNE acceptor, we have produced and characterized four mAbs against HNE-modified proteins (HNEJ-1, 3–5). The results of competitive ELISA assays are summarized in Fig. 2 and Table 1. In contrast to mAb HNEJ-2, which preferentially recognizes histidine adducts as reported previously [19], HNEJ-1 and 3–5 recognized lysine and cysteine adducts to some extent in addition to histidine adducts. HNEJ-1 recognized the three adducts with the same affinity. MAb HNEJ-5 recognized histidine and lysine adducts with approximately the same affinity (Table 1).

3.3. Susceptibility of each protein to modification by HNE

Actin, albumin, glyceraldehyde 3-phophate dehydrogenase (GAPDH), metallothionein and superoxide dismutase (SOD) were incubated in vitro with two different concentrations (1 or 2 mM) of HNE, and analyzed by Western

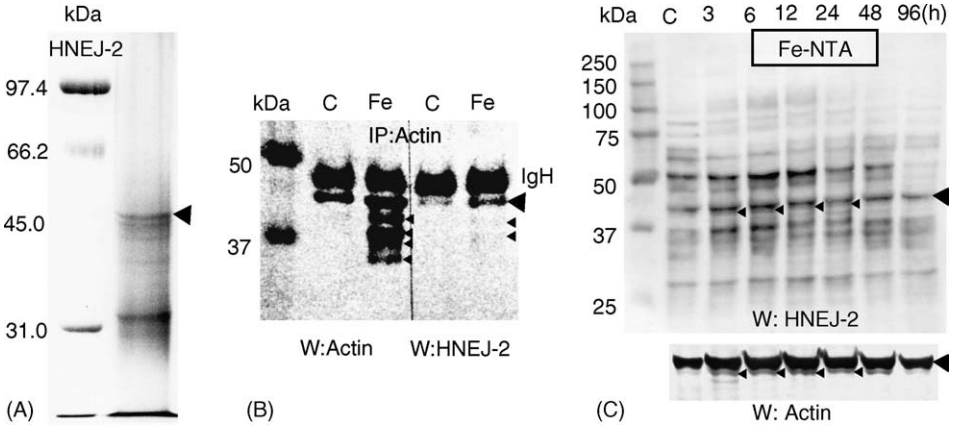

Fig. 1. Identification of actin as a major 4-hydroxy-2-nonenal (HNE)-modified protein in the kidney of rats 3 h after ferric nitrilotriacetate (Fe-NTA) administration. (A) Separation of the cytosolic fraction of kidney samples by affinity chromatography with HNEJ-2 monoclonal antibody, and subsequent analysis by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE); kDa, kilodalton (arrow head, 43 kDa protein). (B) Immunoprecipitation analysis of the cytosolic fraction of kidney samples. Samples were at first immunoprecipitated with anti-actin antibody, and then analyzed by SDS–PAGE with either anti-actin or HNEJ-2 antibody; C, control; Fe, Fe-NTA-treated (3 h); IgH, immunoglobulin heavy chain; IP, immunoprecipitation; W, Western blot analysis (large arrow head, actin at 43 kDa; small arrow head, fragmented actin). (C) Time-course of Western blot analysis after SDS–PAGE of the kidneys of rats after Fe-NTA administration (large arrow head, actin at 43 kDa; small arrow head, minor bands at 41 kDa). Refer to text for details.

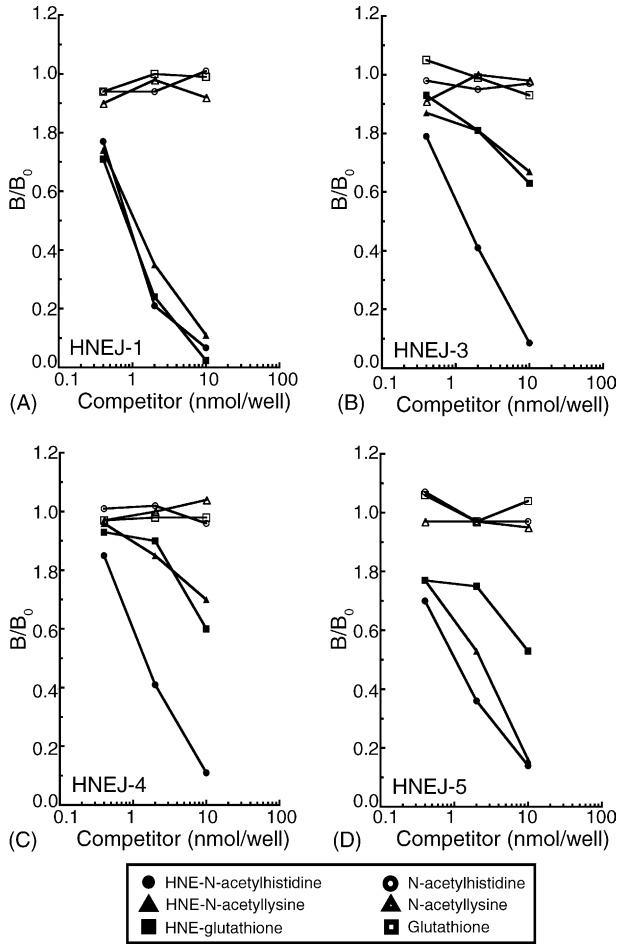

Fig. 2. Characterization of four monoclonal antibodies against HNE-modified proteins with competitive ELISA assays. Assays used HNE-modified glyceraldehyde 3-phosphate dehydrogenase as the adsorbed antigen. The numbers on the abscissa indicate the concentration of competitors as candidate epitopes of Michael adducts (indicated in the box) when antibody was preincubated with competitors at 4 °C for 20 h. B/B_0 was calculated as [experimental OD − background OD (no antibody)]/[total OD (no competitor) − background OD]. Refer to text for details.

blotting using five different mAbs against HNE-modified proteins (HNEJ-1–5). The amount of each protein sample loaded was adjusted to provide the optimal experimental conditions. With Coomassie brilliant blue staining or silver staining, no major changes were observed in the HNE-modified proteins tested (Fig. 3). All the five mAbs recognized the HNE modification. However, the susceptibility of

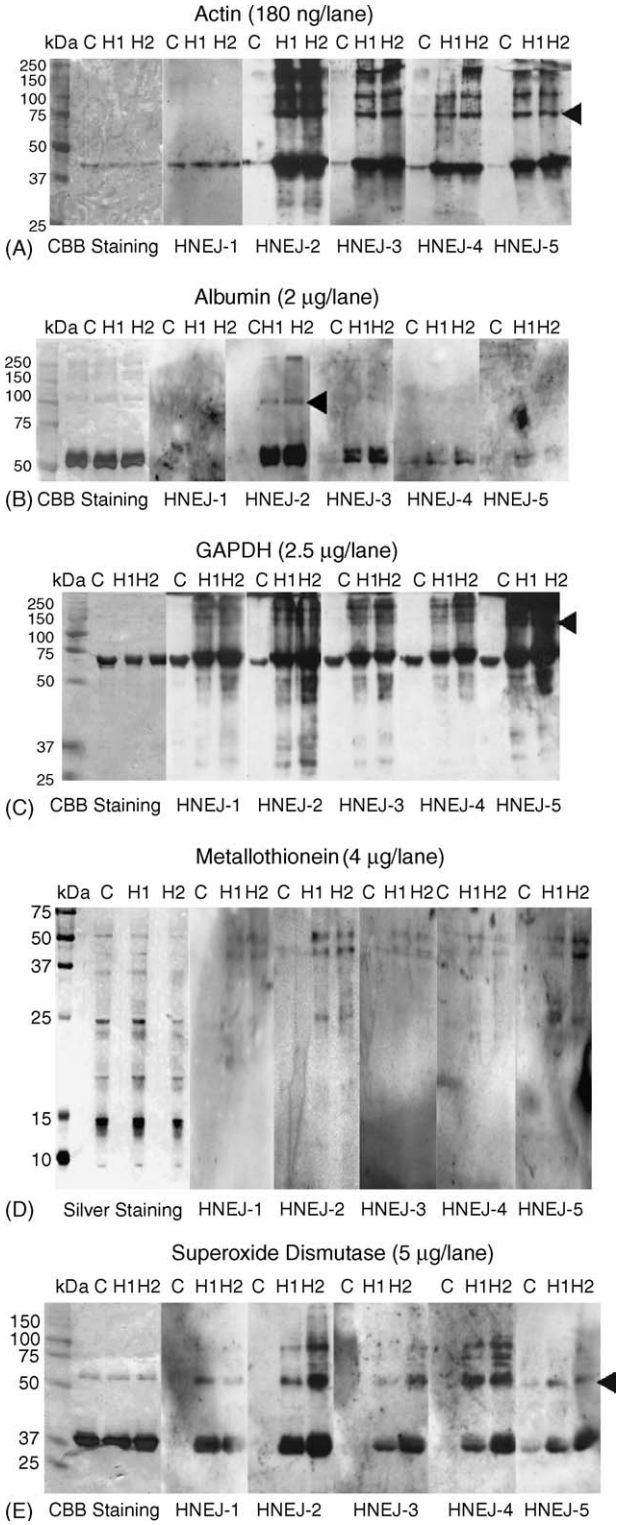

Fig. 3. Western blot analysis of various proteins modified by HNE in vitro. (A) actin; (B) albumin; (C) glyceraldehyde 3-phosphate dehydrogenase; (D) metallothionein; (E) superoxide dismutase. The left-most panel shows Coomasie brilliant blue (CBB) or silver staining after 10–15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The other five panels show the results of Western blot analyses after SDS–PAGE with each monoclonal antibody. Each panel consists of three lanes: C, control; H1, incubated with 1 mM HNE for 1 h; and H2, incubated with 2 mM HNE for 1 h. Compare the original band after CBB or silver staining with the numerous bands after Western blot analyses. Bands with higher molecular weight denote polymerization (arrow head indicates the position of each dimer) whereas bands with lower molecular weight denote fragmentation of the protein. Note that the amount of each protein loaded was adjusted to obtain optimal experimental conditions. HNEJ-1–5 indicates the names of the monoclonal antibodies used. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Refer to Section 3 for further details.

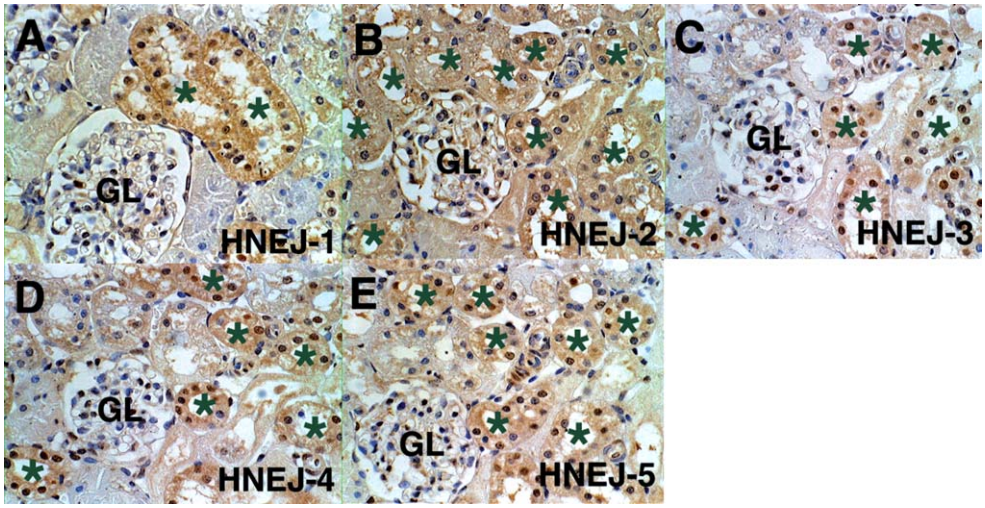

Fig. 4. Immunohistochemistry using monoclonal antibodies against HNE-modified proteins. Kidneys of rats 3 h after Fe-NTA administration were fixed with Bouin's solution, embedded in paraffin and used for immunohistochemical analysis as described in Section 2.1. HNEJ-1–5 indicates the names of the monoclonal antibodies used. Panels A–E are serial sections showing approximately the same areas of the specimen. Immunopositively stained areas are seen as brown color, and the specimens were counterstained with hematoxylin (blue color). GL, glomerulus; *immunopositive renal proximal tubules (original magnification ×200).

each protein to HNE modification and the patterns of recognition were different. Adjustment of the amounts of protein loaded in each lane revealed that actin was most susceptible and metallothionein most resistant to HNE modification among the proteins tested (Fig. 3). Only actin and GAPDH showed detectable HNE modification in the control samples without incubation with HNE. All the proteins showed HNE-modified polymers, whereas only actin and GAPDH showed HNE-modified small fragments (Fig. 3). GAPDH and metallothionein revealed the highest levels of HNE modification by Western blotting with HNEJ-5, whereas the other proteins showed the highest level with HNEJ-2 (Fig. 3).

3.4. Immunohistochemistry of HNE-modified proteins in the kidney

All the mAbs were useful for immunohistochemistry of the paraffin-embedded specimens fixed with Bouin's solution. Immunohistochemistry using mAbs HNEJ-1–5 revealed immunopositivity in the renal proximal tubules 3 h after Fe-NTA administration. However, the patterns of immunostaining differed among the mAbs. HNEJ-2 showed cytoplasmic immunostaining in the majority of proximal tubules, whereas HNEJ-1 or 3–5 revealed cytoplasmic and nuclear immunostaining in a limited area of the proximal tubular cells (Fig. 4A–E).

4. Discussion

In the present study, we identified for the first time one of the major HNE-modified proteins as actin in an oxidative stress-induced carcinogenesis model of rats by the use of HNEJ-2 mAb affinity chromatography. The identification was confirmed by immunoprecipitation and Western lot analyses (Fig. 1B and C). Interestingly, HNE induced fragmentation of actin, and at least two of the fragments were modified with HNE, suggesting that protein inactivation by free radicals had occurred.

Oxidative stress mediated by iron has been shown to be associated with carcinogenesis [27,28]. Fe-NTA-induced renal carcinogenesis presents a unique model to study a molecular mechanism in which produced aldehydes might play an important role [4]. Here we focused on the acute phase of the oxidative renal tubular damage by Fe-NTA in vivo [14]. Thus far, a variety of enzymes have been shown to be susceptible to inactivation by HNE, including glucose-6-phosphate dehydrogenase [29], glutathione S-transferase [30], glutathione reductase [31], interleukin-1β converting enzyme [32] and aldose reductase [33]. However, one of the criticisms of those experiments is that they were all in vitro experiments and the proteins were not selected with a proper screening method in vivo. We found that actin is a major target of HNE modification in vivo. Of note was the fact that HNE-modified actin with various molecular weights was detected as seen in Fig. 1A (∼46 kDa) and C (∼41 kDa). Presumably, higher molecular weight species were caused by massive HNE-modification of actin, which has higher affinity for the column. The presence of a minor band of ∼41 kDa (Fig. 1C) may suggest the presence of a covalent intramolecular conformational change in actin with a faster mobility in the gel.

To further explore the characteristics of actin as an HNE acceptor, we produced and characterized four novel monoclonal antibodies against HNE-modified proteins. These mAbs (HNEJ-1, 3–5) differed from HNEJ-2 mAb in that they reacted with lysine and cysteine adducts, albeit with different affinity (Fig. 2 and Table 1), whereas HNEJ-2 was histidine adduct-specific [19].

We selected four different proteins with distinct localization and function for in vitro HNE modification in comparison with actin. Actin is a cytosolic protein and together with myosin forms the contractile protein complex, actomyosin. In the absence of salt it can exist as a globular protein. However, on the addition of traces of salt, it changes into a highly asymmetrical fibrous protein, F-actin [34]. It was recently reported that HNE induces actin remodeling and barrier function in endothelial cells via mitogen-activated protein kinases pathway [35]. Albumin is a multifunctional serum protein produced by hepatocytes. GAPDH is a cytosolic enzymatic protein associated with glycolysis. Metallothionein is a cytosolic protein that sequesters heavy metals and is associated with detoxification [36]. SOD is a cytosolic enzymatic protein that dismutates superoxide to H_2O_2 and ground-state oxygen [37].

The new four mAbs in combination with HNEJ-2 were helpful in analyzing HNE modification of proteins by Western blot analysis. All the proteins except metallothioneon showed dimers, but further polymers were observed in all the proteins (Fig. 3), revealing a role of HNE in covalent polymerization of proteins. Only actin and GAPDH showed detectable immunoreactivity in the control proteins. This means that these two proteins are prone to oxidative damage during the purification process. Among these proteins, actin was found to be most susceptible to HNE modification, and metallotheionein was most resistant as evaluated by semiquantitative Western blot analysis (Fig. 3). This leads to a speculation that actin may work as a buffer against oxidative stress in vivo, considering the fact that actin is abundant in the cytosol in nearly all kinds of cells.

When comparing actin with metallothionein, both the native proteins are basically linear in configuration, and furthermore, many more target amino acid residues are present in metallotheionein (0 histidine, 6 lysines and 20 cysteines out of 61 amino acids; 42.6%) as compared with actin (9 histidines, 19 lysines, 6 cysteines out of 374 amino acids; 9.1%). The present results should be discussed from the following two standpoints: the characteristics of the mAbs and the characteristics of the proteins. All the mAbs used here were screened during production against HNE-modified albumin that preferentially contains histidine adducts according to the present results (Fig. 3B). Accordingly, all the mAbs might be rather histidine adduct-specific, thus making it difficult to detect cysteine or lysine adducts. However, this possibility may be rejected. The reason is that our recent identification of the modification sites in GAPDH with electrospray ionization liquid chromatography-mass spectrometry showed that Cys-244 and -281, His-164 and -327, and Lys-331 are the target residues [38], and this was consistent with the present finding that mAb HNEJ-5 (recognizing HNE-lysine adducts) showed higher affinity for HNE-modified GAPDH than HNEJ-2. Further, the HNE-resistant tendency of metallothionein was observed for all the mAbs (Fig. 3D). Therefore, we believe that other factors such as metal chelation and covalent polymerizing nature [36] are responsible for the HNE-resistance of metallothionein.

Other interesting finding is that HNEJ-1 which recognizes three types of Michael adduscts in the competitive ELISA assay (Fig. 2A) does not necessarily react strongly with all the adducts in the Western blot analysis (Fig. 3). Consideration of stereoisomers of each Michael adducts may be necessary to explain the results [39]. These mAbs were further used for immunohistochemistry on paraffin-embedded sections with success. The obtained results (Fig. 4A–E) suggest that each mAb reacts with a different set of proteins or other biomolecules in vivo. These differences should be clarified in the near future.

In conclusion, actin is susceptible to HNE-modification in vivo. The four newly characterized mAbs against HNE-modified proteins will be useful for analyzing the nature of HNE modification in vivo.

Acknowledgements

We thank Ms. Waka Kawaguchi for excellent technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

References

[1] E. Shacter, Drug Metab. Rev. 32 (2000) 307.
[2] E. Stadtman, R. Levine, Ann. N.Y. Acad. Sci. 899 (2000) 191.
[3] S. Toyokuni, Pathol. Int. 49 (1999) 91.
[4] K. Uchida, Prog. Lipid Res. 42 (2003) 318.
[5] A. Benedetti, A. Pompella, R. Fulceri, A. Romani, M. Comporti, Biochim. Biophys. Acta 876 (1986) 658.
[6] S. Toyokuni, X.P. Luo, T. Tanaka, K. Uchida, H. Hiai, D.C. Lehotay, Free Radic. Biol. Med. 22 (1997) 1019.
[7] Y. Ebina, S. Okada, S. Hamazaki, F. Ogino, J.L. Li, O. Midorikawa, J. Natl. Cancer Inst. 76 (1986) 107.
[8] J.L. Li, S. Okada, S. Hamazaki, Y. Ebina, O. Midorikawa, Cancer Res. 47 (1987) 1867.
[9] S. Toyokuni, S. Okada, S. Hamazaki, Y. Minamiyama, Y. Yamada, P. Liang, Y. Fukunaga, O. Midorikawa, Cancer Res. 50 (1990) 5574.
[10] S. Toyokuni, Pathol. Int. 49 (1999) 91.
[11] D. Zhang, S. Okada, Y. Yu, P. Zheng, R. Yamaguchi, H. Kasai, Cancer Res. 57 (1997) 2410.
[12] S. Toyokuni, T. Mori, M. Dizdaroglu, Int. J. Cancer 57 (1994) 123.
[13] S. Toyokuni, T. Mori, H. Hiai, M. Dizdaroglu, Int. J. Cancer 62 (1995) 309.
[14] S. Toyokuni, K. Uchida, K. Okamoto, Y. Hattori-Nakakuki, H. Hiai, E.R. Stadtman, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 2616.
[15] K. Uchida, A. Fukuda, S. Kawakishi, H. Hiai, S. Toyokuni, Arch. Biochem. Biophys. 317 (1995) 405.
[16] T. Tanaka, Y. Iwasa, S. Kondo, H. Hiai, S. Toyokuni, Oncogene 18 (1999) 3793.
[17] M. Hiroyasu, M. Ozeki, H. Kohda, M. Echizenya, T. Tanaka, H. Hiai, S. Toyokuni, Am. J. Pathol. 160 (2002) 419.
[18] T. Tanaka, S. Akatsuka, M. Ozeki, T. Shirase, H. Hiai, S. Toyokuni, Oncogene 23 (2004) 3980.
[19] S. Toyokuni, N. Miyake, H. Hiai, M. Hagiwara, S. Kawakishi, T. Osawa, K. Uchida, FEBS Lett. 359 (1995) 189.

[20] L. De Montarby, P. Mosset, R. Gree, Tetrahedron Lett. 29 (1988) 3895.
[21] E. Schauenstein, M. Taufer, H. Esterbauer, A. Kylianek, T. Seelich, Monatch. Chem. 102 (1971) 517.
[22] K. Uchida, L.I. Szweda, H.-Z. Chae, E.R. Stadtman, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8742.
[23] L.G. Luna, Manual of histological staining methods of the Armed Forces Institute of Pathology, McGraw-Hill, New York, 1968.
[24] H. Kaneshima, M. Ito, J. Asai, O. Taguchi, H. Hiai, Lab. Invest. 56 (1987) 372.
[25] S. Toyokuni, W. Kawaguchi, S. Akatsuka, M. Hiroyasu, H. Hiai, Pathol. Int. 53 (2003) 259.
[26] S. Toyokuni, T. Tanaka, Y. Hattori, Y. Nishiyama, H. Ochi, H. Hiai, K. Uchida, T. Osawa, Lab. Invest. 76 (1997) 365.
[27] S. Toyokuni, Free Radic. Biol. Med. 20 (1996) 553.
[28] T. Wu, C. Sempos, J. Freudenheim, P. Muti, L. Smit, Ann. Epidemiol. 14 (2004) 195.
[29] L.I. Szweda, K. Uchida, L. Tsai, E.R. Stadtman, J. Biol. Chem. 268 (1993) 3342.
[30] A. Mitchell, D. Morin, M. Lame, A. Jones, Chem. Res. Toxicol. 8 (1995) 1054.
[31] D. Vander Jagt, L. Hunsaker, T. Vander Jagt, M. Gomez, D. Gonzales, L. Deck, R. Royer, Biochem. Pharmacol. 53 (1997) 1133.
[32] D. Davis, R. Hamilton, A. Holian, J. Interferon Cytokine Res. 17 (1997) 205.
[33] A. Del Corso, M. Dal Monte, P. Vilardo, I. Cecconi, R. Moschini, S. Banditelli, M. Cappiello, L. Tsai, U. Mura, Arch. Biochem. Biophys. 350 (1998) 245.
[34] W. Kabsch, H. Mannherz, D. Suck, E. Pai, K. Holmes, Nature 347 (1990) 37.
[35] P. Usatyuk, V. Natarajan, J. Biol. Chem. 279 (2004) 11789.
[36] J. Kagi, Y. Kojima, Exp. Suppl. 52 (1987) 25.
[37] B. Halliwell, J.M.C. Gutteridge, Free radicals in Biology and Medicine, Clarendon Press, Oxford, 1999.
[38] T. Ishii, E. Tatsuda, S. Kumazawa, T. Nakayama, K. Uchida, Biochemistry 42 (2003) 3474.
[39] M. Hashimoto, T. Sibata, H. Wasada, S. Toyokuni, K. Uchida, J. Biol. Chem. 278 (2003) 5044.