The monoclonal antibody specific for the 4-hydroxy-2-nonenal histidine adduct

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Abstract **Monoclonal antibodies directed against proteins modified with the major membrane lipid peroxidation product, 4-hydroxy-2-nonenal, have been established and characterized. The monoclonal antibodies specific for HNE-modified proteins were raised by immunizing mice with a HNE–keyhole limpet hemocyanin conjugate. The resulting five monoclonal antibodies (mAbs HNEJ-1–5) recognized HNE-modified bovine serum albumin (BSA), but not native BSA in Western blot studies. Of the five mAbs, HNEJ-2 exhibited the highest affinity for HNE-modified proteins and a much higher affinity for the HNE–histidine adduct than the HNE–lysine or HNE–cysteine adducts. mAb HNEJ-2 did not cross-react with proteins that had been treated with other aldehydes, such as 1-hexenal, 2-hexenal, 4-hydroxy-2-hexenal, 2-nonenal, formaldehyde, or glutaraldehyde. These results suggest that the major epitope recognized by mAb HNEJ-2 is the Michael addition-type HNE–histidine adduct.**

Key words: Reactive oxygen species; Lipid peroxidation; 4-Hydroxy-2-nonenal; Histidine; Monoclonal antibody

1. Introduction

Reactive oxygen species (ROS) are involved in a diversity of biological phenomena, such as inflammation, mutagenesis, carcinogenesis, aging, atherosclerosis, and ischemia–reperfusion injury [1]. Membrane lipids are one of the major targets of ROS, initiating the free radical chain reaction by lipid peroxidation [1]. In this process, a variety of aldehydes are generated as final products when lipid hydroperoxides break down. Among them, 4-hydroxy-2-nonenal (HNE) is an α,β-unsaturated aldehyde that can be formed by peroxidation of ω6-unsaturated fatty acids such as linoleic and arachidonic acids. In biological systems, HNE originates almost exclusively from phospholipid-bound arachidonic acid, and may be the most reliable marker of lipid peroxidation [2].

HNE exhibits various cytopathological effects, such as enzyme inhibition, inhibition of DNA, RNA and protein synthesis, and induction of heat-shock proteins [2]. It is highly toxic to many types of cells, including hepatocytes, fibroblasts and Ehrlich ascites tumor cells. HNE also exhibits genotoxic and mutagenic effects, as well as inhibitory effects on cell proliferation [2]. In a model system using Ehrlich ascites tumor cells, more than 80% of HNE are metabolized to non-toxic compounds, either by oxidation or reduction, within 10 min after exposure. However, a fraction (approximately 1–6%) of HNE is covalently bound to proteins [3]. It has been proposed that HNE exerts the cytotoxic effects via its facile reactivity with proteins. We have previously established that the imidazole nitrogens of the histidine residue is one of the major targets of HNE reactivity, in addition to the ε-amino group of the lysine residue and the sulfhydryl group of cysteine residue [4].

We have recently raised a polyclonal antibody against HNE-modified proteins, and showed that epitopes recognized by this polyclonal antibody are present in oxidized cultured hepatocytes [5], atherosclerotic lesions of human aorta [6], renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate [7], and human renal cell carcinoma [8]. However, polyclonal antibodies recognize multiple epitopes and have a limitation in the quantity that can be prepared. In the present study, we established and characterized five monoclonal antibodies (mAbs) specific for HNE-modified proteins.

2. Materials and methods

2.1. Materials

The stock solution of *trans*-4-hydroxy-2-hexenal and *trans*-4-hydroxy-2-nonenal were prepared by acid treatment (1 mM HCl) of HNE diethylacetal, which was synthesized according to the procedure of De Montarby et al. [9]. The concentration of the HNE stock solution was determined by measurement of UV absorbance at 224 nm [10]. 2-Nonenal, 1-hexenal, glutaraldehyde, and formaldehyde were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Keyhole limpet hemocyanin (KLH) was obtained from Pierce (Rockford, IL). Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin was obtained either from Dako (Kyoto, Japan) or Amersham (Little Chalfont, England). Enhanced chemiluminescence (ECL) immunoblotting detection reagents were obtained from Amersham. Bovine serum albumin (BSA) was obtained from Intergen Co. (Purchase, NY). 2-Hexenal, Nα-acetyl-L-histidine, Nα-acetyl-L-lysine and glutathione were obtained from Sigma (St. Louis, MO). Protein concentration was measured using the BCA protein assay reagent obtained from Pierce.

2.2. Immunization

The HNE-modified KLH (HNE–KLH) immunogen was prepared by reacting KLH with HNE as previously described [5]. The HNE-modified BSA (HNE–BSA) was prepared by incubating 10 mg/ml of BSA with 1 mM HNE at 37°C for 2 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 vol., respectively) to female BALB/c mice (7 weeks of age; Shizuoka Laboratory Center, Shizuoka, Japan). After 4 and 8 weeks, booster injections in incomplete Freund's adjuvant (Difco) and without adjuvant, respec-

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Abbreviations: ROS, reactive oxygen species; HNE, 4-hydroxy-2-nonenal; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline.

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tively, were done. The antibody response in serum was monitored by Western blot and ELISA using BSA and HNE–BSA as the paired antigens.

2.3. Monoclonal antibody

The fusion was performed by the use of 8-azaguanine-resistant murine myeloma P3X63-Ag8.653 (653) cells at a ratio of 5:1 as previously described [10]. 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 by ELISA using BSA and HNE–BSA as the coupled antigens. mAbs were purified by ammonium sulfate from ascites of nude mice (BALB/c, nu/nu, female, 8 weeks of age) after intraperitoneal injections of 10^7 hybridoma cells as previously described [11]. Immunoglobulin subclass was determined by a kit from Amersham.

2.4. ELISA

For monitoring antibody response and cloning hybridomas, 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 in PBS containing 1% BSA, 0.5% Tween 20, each well was filled with 200 μl PBS/BSA to block non-specific binding for 1 h at room temperature (RT). After three washes, 150 μl of the serum (diluted 1:100) or culture supernatant (diluted 1:3) was added to each well and 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 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 incubated for several minutes at RT. The reaction was terminated by addition of 2 M sulfuric acid and was quantified by absorbance at 490 nm with a microtiter plate reader (M-EMax; Molecular Devices, Menlo Park, CA). For studies of HNE specificity and competition, aldehyde-modified proteins and the haptens (HNE–*N*-acetylhistidine, HNE–*N*-acetyllysine, and HNE–glutathione) were prepared and ELISA carried out as previously described [5].

2.5. Western blot analysis

Western blot analyses were done using 12.5% SDS-PAGE gels as previously described [5]. The bands were visualized by ECL reagents and autoradiography.

3. Results

3.1. Hybridomas secreting antibodies against HNE-modified proteins

Spleen cells of the immunized mice were fused with 653 cells and seeded into 720 wells of microwell plates. By screening 320 hybridoma colonies, 28 highly positive colonies ($A_{HNE\text{-}BSA}/A_{BSA} > 20$) were obtained. We selected 5 colonies based on proliferating activity, and cloned 5 hybridomas by repeated limiting dilutions. The 5 cloned hybridomas were named HNEJ-1–5, respectively. The culture medium of HNEJ-1, -3 and -4 was changed to CM-B (Sanko Pharmaceutical, Tokyo, Japan) to maintain high proliferating activity.

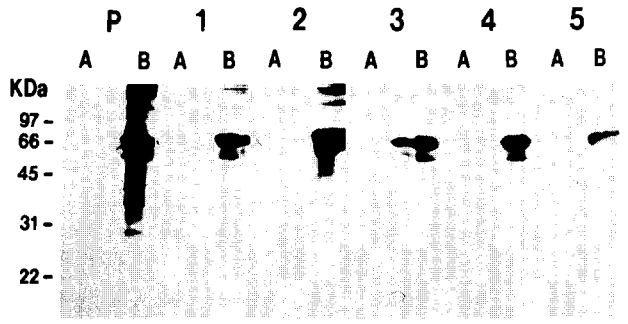

Fig. 1. Western blot analysis of mAbs HNEJ-1–5 (200 μg/ml) using BSA and HNE–BSA (25 μg each/lane). (A) BSA; (B) HNE–BSA; (P) polyclonal antibody published in [5] (2 μg/ml) as a control. 1–5 correspond to mAbs HNEJ-1–5, respectively.

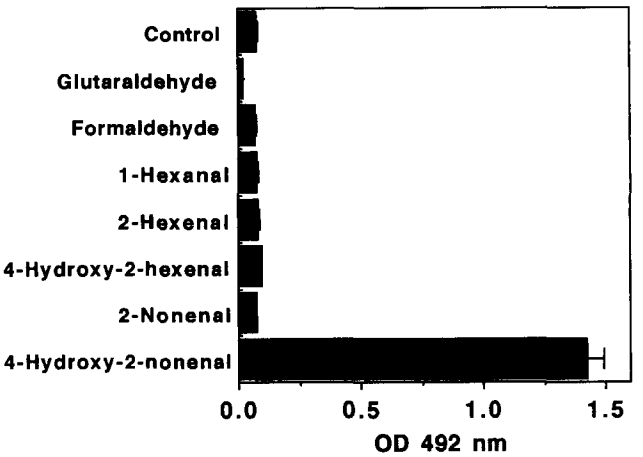

Fig. 2. ELISA study for the specificity of mAb HNEJ-2. Aldehyde-modified proteins were prepared by reacting protein (GAPDH) with 1 mM aldehyde (1 M for glutaraldehyde and formaldehyde) in 50 mM sodium phosphate buffer (pH 7.2) for 2 h at 37°C. Refer to section 2 for details.

3.2. Characterization of the mAbs

All five antibodies were IgG1, κ subtype. The specificity of each antibody was studied by Western blot analysis. mAbs HNEJ-1–5 (200 μg/ml) recognized HNE–BSA but not BSA by Western blot (Fig. 1). The order of affinity for HNE–BSA was (mAb HNEJ-2 > 1 = 3 = 4 > 5). mAb HNEJ-2 was further characterization using ELISA. As shown in Fig. 2, mAb HNEJ-2 showed almost negligible reactivity with proteins that had been treated with other aldehydes, such as 2-nonenal, 2-hexenal, 1-hexenal, 4-hydroxy-2-hexenal, formaldehyde, or glutaraldehyde.

3.3. Competition by HNE–amino acid adducts

As shown in Fig. 3, among the HNE adducts (*N*-acetylhistidine, *N*-acetyllysine and HNE–glutathione) tested, only HNE–*N*-acetylhistidine inhibited the binding of antibody to the HNE-modified protein. In a control study the non-HNE-derivatized forms of these compounds exhibited no effect.

4. Discussion

We have previously prepared a polyclonal antibody against HNE-modified proteins [5] and demonstrated that the antibody is quite useful for the evaluation of ROS-induced damage both in vitro and in vivo [5–8]. This prompted us to raise monoclonal antibodies against HNE-modified proteins. We obtained 5 independent mAbs by repeated cloning. All mAbs were highly specific for HNE-modified proteins. Indeed, mAb HNEJ-2 did not recognize 4-hydroxy-2-hexenal-modified proteins solely by the difference in carbon chain length. In addition, mAb HNEJ-2 did not recognize glutaraldehyde- or formaldehyde-modified proteins (Fig. 2). This is advantageous for the application of

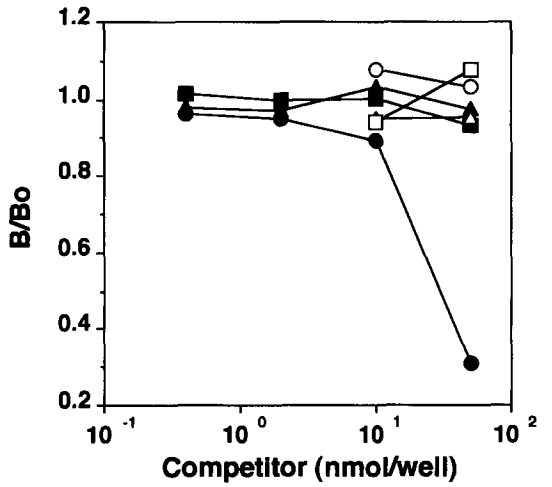

Fig. 3. ELISA competition curves for mAb HNEJ-2. Refer to section 2 for details. Assays used HNE-modified GAPDH as the absorbed antigen. The numbers on the abscissa indicate the concentration of competitors when antibody was preincubated with competitors at 4°C for 20 h. Competitors were as follows: ●, HNE–*N*-acetylhistidine; ▲, HNE–*N*-acetyllysine; ■, HNE–glutathione; ○, *N*-acetylhistidine; △, *N*-acetyllysine; □, glutathione. B/B_o, calculated as [experimental OD − background OD (no antibody)]/[total OD (no competitor) − background OD].

this mAb to immunohistochemistry since glutaraldehyde and formaldehyde are commonly used for morphological fixation. Further characterization of the other four mAbs are now in progress. The affinity of the mAbs for HNE-modified proteins was lower than that of the polyclonal antibody. This is probably because the polyclonal antibody recognizes multiple epitopes surrounding Michael adduct structures.

By inhibition tests, we unexpectedly found that mAb HNEJ-2 has a much higher affinity for the HNE–histidine adduct than the HNE–lysine or HNE–cysteine adduct. This implies that there is a slight structural difference between Michael addition-type HNE–histidine adducts and HNE–lysine or HNE–cysteine adducts, and that the histidine–HNE adduct is a distinctive epitope for immune recognition. Based on our results it is stressed that the HNE–histidine Michael addition reaction that we have previously reported [4] is unique and important in biology.

In summary, we report for the first time the preparation and characterization of mAbs for HNE-modified proteins. We believe that these mAbs will be useful for the evaluation of ROS-induced damage, not only in lipids in industry and food but also in membrane damage in various biological systems both in vitro and in vivo.

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