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Immunochemical Detection of Protein Dityrosine in Atherosclerotic Lesion of Apo-E-Deficient Mice Using a Novel Monoclonal Antibody

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Dityrosine is one of the specific biomarkers for protein oxidation. We prepared an antibody specific for protein dityrosine using a dimer of 3-*p*-(hydroxyphenyl)propionic acid (di-HP) as a hapten. Three clones (A8, G6, and 1C3) were obtained, and the antibody from the A8 clone reacted with the di-HP-conjugated protein but not with a dityrosine conjugate. The others (G6 and 1C3 clones) recognized both the di-HP and dityrosine conjugates. The antibodies reacted with peptidyl dityrosine, derived from Thr-Tyr-Ser, rather than the free dityrosine. The reactivity of the latter two antibodies with lens proteins oxidized by incubation with H_2O_2/Cu was in good accordance with the formation of the dityrosine-like fluorescence. Using the obtained monoclonal antibody, the immunopositive staining of atherosclerotic lesions in Apo E-deficient mice was confirmed by an immunohistochemical technique.

Key Words: **dityrosine; monoclonal antibody; oxidation; atherosclerosis; Apo E-deficient mouse.**

Dityrosine is a fluorescent dimer of tyrosine and is formed by reactive oxygen species (1), ultraviolet irradiation (2), and peroxidases (3, 4). The isolation and identification in connective tissue proteins such as collagen in tendons or the skin, and elastin in the aorta of chick embryos have been reported (5–8). The presence of dityrosine bridges in thyroglobulin, the prothyroid hormone, has also been confirmed (9–11). The detection of dityrosine in atherosclerotic lesions was also reported (12, 13).

Antibodies to oxidative stress markers have been widely used, and the immunological methods have many advantages in the study of the localization of the markers, whereas these methods are semiquantitative. We focused on dityrosine as one of the biomarkers for oxidative protein damage, and the specific polyclonal antibody has already been prepared (14). However, the polyclonal antibody was not suitable for the estimation of dityrosine buried in protein due to its preferential specificity for free dityrosine more than for peptidyl dityrosine. We have improved the preparation protocol to obtain a monoclonal antibody recognizing protein dityrosine using a novel immunogen. In this report, the specificity of three new obtained monoclonal antibodies is shown. Using the antibody, the formation of protein dityrosine in atherosclerotic lesions of Apo E-deficient mice has been estimated.

MATERIALS AND METHODS

Materials. Dimethylformamide, *N*-hydroxysucciniimide, hydrogen peroxide (H_2O_2, 30%), and 3-(*p*-hydroxyphenyl)propionic acid (HP) were obtained from the Wako Pure Chemical Co. Horseradish peroxidase (HRP), *Arthromyces* peroxidase, catalase, and lens proteins (from bovine eye lens: water soluble) were purchased from the Sigma Chemical Co. Keyhole limpet hemocyanin (KLH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from the Pierce Chemical Co.

Preparation of immunogen. The dimer of 3-(*p*-hydroxyphenyl) propionic acid (HP) was prepared by incubation of HP with HRP in the presence of H_2O_2. Briefly, HP (0.17 g), HRP (3 mg), and 30% H_2O_2 (120 μl) in 200 ml of 0.01 M borate buffer (pH 9.0) were incubated at 37°C for 2 h. The reaction was terminated by the addition of 5 μl (20,000 units) catalase and then further incubated for 15 min. The product was concentrated, and then applied to an HW-40 gel filtration open column, which had been equilibrated with water. The fractions having fluorescence (ex. 300 nm/em. 400 nm) were collected and concentrated. Aliquots of the fractions were simultaneously analyzed by reversed-phase high performance liquid chromatography (HPLC), and it was confirmed that a single peak (detected at UV 280 nm), which has the fluorescence nature of the dihydroxyphenyl moiety, was included in the fractions. The liquid chromatography-mass spectrometry (LC-MS) analysis of the fraction revealed that the

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product showed 329 $(M-H)^-$ as the parent ion. Based on these results, the product was identified as a dimer of 3-(*p*-hydroxyphenyl)propionic acid, named di-HP.

The obtained dimer (di-HP) was conjugated with KLH and BSA using EDC and *N*-hydroxysuccinimide. Briefly, the dimer (16 mg), EDC (18 mg), and *N*-hydroxysuccinimide (20 mg) were dissolved in 1.6 ml of dimethylformamide and reacted overnight at room temperature with stirring. The reaction mixture was then equally divided. One part was mixed with 30 mg of lipid-free BSA in 3.8 ml of phosphate buffer (pH 7.4), and the other was mixed with 20 mg of KLH in 3.8 ml of the phosphate buffer at room temperature with stirring for 4 h. The modified proteins (di-HP-KLH and di-HP-BSA) were dialyzed against water at 4°C for 2 days with several exchanges of water.

HPLC and LC-MS of di-HP. The sample was injected into a Develosil ODS-HG-5 column (4.6 × 150 mm) and eluted with 0.1% acetic acid/CH3CN (1/1) at a flow rate of 0.8 ml/min. The elution was monitored by a UV/Vis-detector (280 nm) and a fluorescence detector (ex. 300 nm/em. 400 nm). For the LC-MS, the separation by HPLC was performed under the same conditions. The detection was performed by a PLATFORM II (VG Biotech) mass spectrometer using the electrospray ionization (positive) mode.

Preparation of dityrosine-conjugated BSA. Dityrosine-conjugated BSA (DT-G-BSA) was prepared by incubation of lipid-free BSA and dityrosine with glutaraldehyde as already described (14). Dityrosine was synthesized enzymatically and purified as previously described (2).

Immunization and antibody preparation. Balb/c mice were intraperitoneally primed with the modified KLH (di-HP-KLH, 75 μg/mouse) emulsified in Freund's complete adjuvant. Mice were boosted twice at a 2-week interval. After 8 weeks, the animals received an intraperitoneal final injection of the antigen without adjuvant. The production and selection of hybridomas were carried out by the method of Kohler and Milstein (15) with a slight modification. The (positive) clones were selected using the di-HP-BSA and/or dityrosine-conjugated BSA (DT-G-BSA) as the antigen for the enzyme-linked immunosorbent assay (ELISA). The obtained hybridomas were intraperitoneally injected into Balb/c mice, which had been injected with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane) one week before and, about 7–10 days later, the ascites were collected by laparotomy. Partially purified antibodies were prepared using ammonium sulfate fractionation and used for the experiments.

Oxidative modification of proteins with H_2O_2/Cu. Proteins (0.5 mg/ml) were incubated with H_2O_2 (5 mM) and $CuSO_4$ (0.05 mM) in a 0.1 M phosphate buffer (pH 7.4) at 37°C for 0–24 h. The reaction was terminated by adding 0.1 mM ethylenediaminetetraacetic acid (EDTA). The reaction mixture was stored at −20 or −70°C until use.

Fluorescence measurements. The reaction mixture was used for the fluorescence measurement (ex. 300 nm/em. 400 nm) along with a Hitachi F-2000 fluorescence spectrometer.

Enzyme-linked immunosorbent assay (ELISA). The indirect ELISA was performed as follows: Briefly, for the time-course experiments, 50 μl of modified proteins (0.01 mg/ml in phosphate buffered saline (PBS) containing 0.1 mM EDTA was dispensed into wells and kept overnight at 4°C. After the coating, the plate was washed with PBS containing 0.25% Tween 20 (TPBS) and water, and 2% blocking agent (Block Ace, Dainihon Seiyaku, Osaka, Japan) was then added and further incubated for 1 h at 37°C. The plate was then incubated with the monoclonal antibody (0.1–0.2 μg/ml TPBS) for 2 h at 37°C, and the binding of the antibody to the modified protein was evaluated by incubation with a peroxidase-labeled anti-mouse IgG antibody (1/5000). The color development was performed by reaction of the conjugated peroxidase with *o*-phenylenediamine and H_2O_2. The data represent the mean of triple determinations.

The cross-reactivity with antibody was also investigated by indirect competitive ELISA: As a coating agent, 50 μl of di-HP-BSA (0.1–0.5 μg/ml PBS) was pipetted into wells and kept overnight at 4°C. At the same time, 50 μl of antibody (0.05–0.1 μg/ml TPBS) and 50 μl of sample were mixed in an Eppendorf tube and reacted overnight at 4°C. The plate was washed, blocked, and 90 μl of the reacted solution was then pipetted into a well. The binding of the residual antibody on coated modified BSA was estimated as described above. The result of competitive ELISA was expressed as B/B0, where B is the amount of antibody bound in the presence and B0 the amount in the absence of the competitor. Each point represents the mean of duplicate determinations.

Animals. The Apo E-deficient mutant strain was developed in the laboratory of Dr. Nobuyo Maeda at the University of North Carolina at Chapel Hill (16). The Apo E-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Immunohistochemical analysis. Tissue sections (5 μm) were prepared from the paraffin embedded aortic arch of 6-month-old male Apo E-deficient mice fed normal chow. Sections were immunostained with a Vectrastain ABC-AP (alkaline phosphatase) commercial kit (Funakosi, Tokyo, Japan). Sections were incubated with normal serum in PBS for 20 min to block nonspecific binding before staining and then with the G6 monoclonal antibody (1:1000 dilution) or 5% normal mouse serum IgG instead of the primary antibody as the negative control. Immunostaining was performed with biotinic affinity refined immunogloblin (1:200 dilution) as the second antibody and with the reagent avidin DH plus biotinic alkaline phosphatase H in a ratio of 1:1 as the enzymatic marker and with Vector Red commercial kit as the chromogen.

RESULTS

Preparation of a Novel Immunogen and the Reactivity with Polyclonal Antibody

We have already reported the preparation of a polyclonal antibody to dityrosine using the dityrosine-conjugated keyhole limpet hemocyanin (14). The polyclonal antibody recognized free dityrosine but not the other tyrosine-related compounds. However, a weak binding ability to protein dityrosine was assumed by comparison of the peptidyl dityrosine with free dityrosine. This result suggested that free dityrosine was probably attached to the surface of the carrier protein like the protein tyrosylation. To prepare the antibody recognizing the protein dityrosine (intra- and intermolecular protein-bound dityrosine), a dityrosine-like compound, the dimer of 3-(*p*-hydroxyphenyl)propionic acid (di-HP), was used as a hapten because of effective conjugation. The 3-(*p*-hydroxyphenyl)propionic acid (HP) was treated with HRP in the presence of H_2O_2, and the formed dimer (di-HP) was then conjugated with KLH and used for the immunogen. At first, we examined the reactivity of the polyclonal antibody to the di-HP conjugated KLH (immunogen) and its analog (di-HP-BSA) to check the antigen preparation. The polyclonal antibody to dityrosine could also recognize the novel synthetic conjugates. These results suggested that the epitopes of the polyclonal antibody resembled the di-HP moiety.

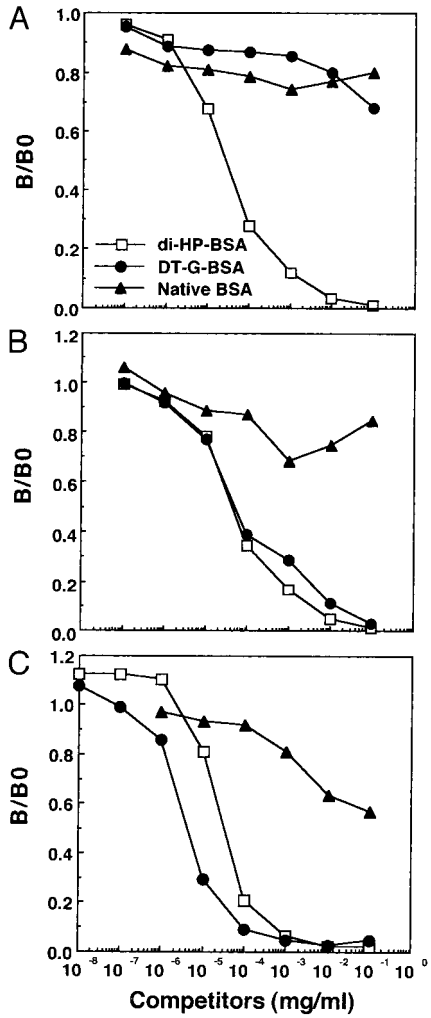

FIG. 1. Competitive ELISA of synthetic hapten- and dityrosine-conjugated proteins. The hapten (a dimer of 3-(*p*-hydroxyphenyl) propionic acid) was conjugated with BSA using carbodiimide and the conjugate (di-HP-KLH) was then dialyzed. Dityrosine-conjugated BSA (DT-G-BSA) was prepared by incubation of lipid-free BSA and dityrosine with glutaraldehyde. The cross-reactivity of the antibodies ((A) A8 clone, (B) G6 clone, and (C) 1C3 clone) with the proteins was investigated by indirect competitive ELISA using di-HP-BSA as a coating agent as described under Materials and Methods.

The Production of Monoclonal Antibodies and Competitive ELISA with Dityrosine Conjugated Protein

The novel immunogen, modified KLH, was injected into mice, and monoclonal antibodies were prepared by the fusion of myeloma cells with spleen cells using polyethylene glycol according to the standard protocol. The generation of the antibody to the dihydroxyphenyl moiety was estimated by ELISA using both DT-G-BSA and di-HP-BSA. As a control, the hybridoma (named A8 clone), which generates the antibody to di-HP-BSA but not DT-G-BSA, was also selected. Finally, three clones (A8, G6, and 1C3), which secreted immunoglobulin G, were obtained. The results of the competitive ELISA using these clones are shown in Fig. 1. The antibody from the A8 clone reacted with the di-HP-conjugated protein but not with the dityrosine conjugate (Fig. 1A). The others (G6 and 1C3 clones) recognized both the di-HP and dityrosine conjugates (Figs. 1B and C).

Estimation of Epitopes by Competitive ELISA Using Low Molecular Weight Compounds as Competitors

The monoclonal antibodies (G6 and 1C3) react with free dityrosine, whereas the other tyrosine related compounds, such as tyrosine, phenylalanine, 3-nitrotyrosine, and 3-chlorotyrosine, could not be recognized by the antibody. The antibody reacted with dityrosine in the dimer of Thr-Tyr-Ser rather than with the free dityrosine, suggesting preferential recognition of the antibody to the protein dityrosine compared to the free dityrosine (Fig. 2).

Application of the Antibodies to Oxidatively Modified Proteins

The time-dependent formation of immunoreactivity against lens proteins exposed to H_2O_2/Cu was observed (Fig. 3A). The increase in fluorescence of the proteins was consistent with the increase in the immunoreactivity (Fig. 3C). These results suggested that the immunoreactivity should be due to the formation of the dityrosine moiety in the proteins. The reactivity of the polyclonal antibody with oxidized lens proteins was very weak compared to the monoclonal antibody (Fig. 3B). These results coincided with the preferential specificity of the polyclonal antibody for free dityrosine but not for peptidyl dityrosine (protein dityrosine) (14). In addition, the antibody secreted from the G6 clone also

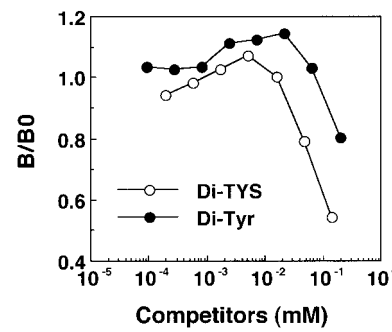

FIG. 2. The antibody recognized the peptidyl dityrosine rather than the free dityrosine. The dimer of Thr-Tyr-Ser (Di-TYS) was prepared using *Arthromyces* peroxidases in the presence of H_2O_2 and Thr-Tyr-Ser as previously described (14). The presence of the dityrosine moiety in the dimer was confirmed by reversed phase HPLC connected to a fluorescence detector, following acid hydrolysis of the isolated Di-TYS. The ELISA using the 1C3 clone was performed as described in the legend of Fig. 1. The antibody from the G6 clone showed similar results.

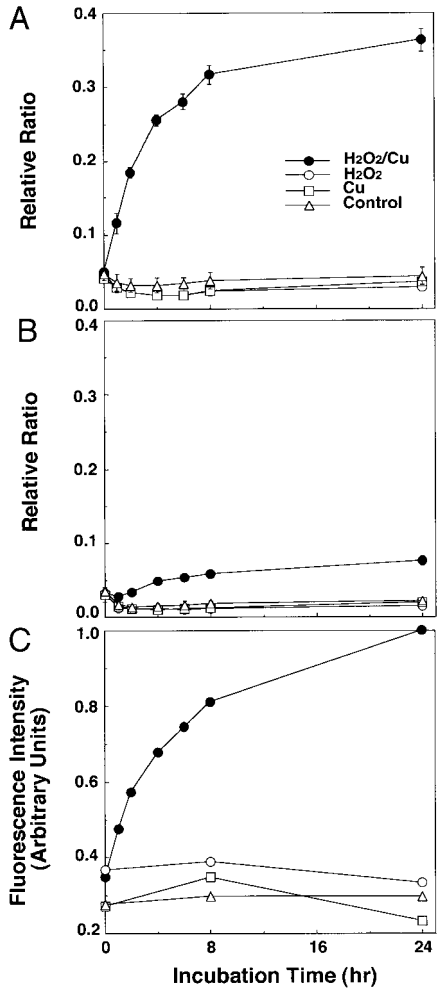

FIG. 3. The time-dependent formation of the immunoreactivity with H_2O_2/Cu was observed. The lens proteins (0.5 mg/ml) was incubated with/without 5 mM H_2O_2 and/or 0.05 mM $CuSO_4$ in a phosphate buffer (pH 7.4) at 37°C. The reaction was terminated by adding 1 mM ethylenediaminetetraacetic acid (EDTA) at various intervals. ELISA was performed as described under Materials and Methods. The ELISA data was expressed by comparison with positive controls (DT-G-BSA 0.5 μg/ml) as a relative ratio. "Control" means incubation of protein only. (A) The immunoreactivity against the monoclonal antibody (1C3) measured by noncompetitive ELISA. (B) The immunoreactivity against polyclonal antibody measured by noncompetitive ELISA. (C) The fluorescence intensity (ex. 300 nm/em. 400 nm) of the reaction mixture measured using a fluorescence spectrophotometer.

reacted with the oxidized proteins similar to that from the 1C3 clone. The antibody from the A8 clone (di-HP specific) could not recognize these oxidatively-modified proteins.

Immunohistochemical Study

The detection of dityrosine in an atherosclerotic lesion was already reported using chemical methods by HPLC or gas chromatography-mass spectrometry (12, 13). Therefore, we examined the immunohistochemical staining of dityrosine using the monoclonal antibody in an atherosclerotic lesion of Apo E-deficient mice. As shown in Figs. 4A and 4C, the positive staining in the fatty streak was observed.

DISCUSSION

Dityrosine is formed via tyrosyl radical intermediates. The tyrosyl radical can cause lipid peroxidation via radical reactions (H-abstraction of the lipid moiety) (17) and the participation of myeloperoxidase in the dityrosine formation has been suggested (12, 18). Though dityrosine was already detected from atherosclerotic lesions (21, 22), the localization of dityrosine in the fatty streak of atherosclerotic plaque in Apo E-deficient mice was visually revealed by an immunohistochemical method using the novel monoclonal antibody recognizing free and protein dityrosines (Fig. 4). In the experiment, the avidin-biotin-alkali phosphatase complex was used for the development to avoid the possibility of contribution of internal peroxidase activity to the staining because peroxidases can generate dityrosine (3, 4). In addition, after the treatment of the sections with hydrogen peroxide to inactivate (plausible) internal peroxidases, positive staining was also

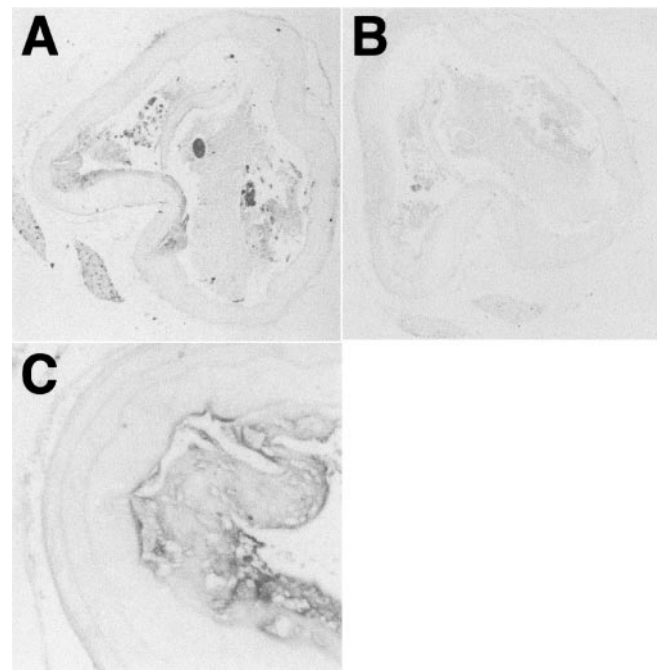

FIG. 4. Photomicrographs showing positive immunostaining of protein dityrosine in the sections of aortic arch of a 6-month-old male Apo E-deficient mice fed normal chow. A (×40) & C (×200), sections stained with the G6 antibody (1:1000 dilution), specific epitopes recognized by the primary antibody are shown in the fatty streak. B (×40), no staining was observed in the section stained with 5% normal mouse serum IgG instead of the primary antibody as the negative control.

observed using avidin-biotin-peroxidase development (data not shown).

It has been reported that the caloric restriction attenuated the dityrosine contents in the cardiac and skeletal muscles of mice (19). The detection/quantification of dityrosine would become a biomarker of "pure" protein oxidation. "Pure" means that the formation of dityrosine is not generated by lipid-derived and glycation-derived aldehydes. The nonimmunochemical methods such as gas chromatography-mass spectrometry or HPLC-electrochemical detection are suitable for the "correct" (net) quantitation of dityrosine, whereas these approaches for the detection of dityrosine require several sample preparation steps. The immunochemical methods should be useful techniques for dityrosine detection in cells and tissues with simple procedures.

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