Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain

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Abstract Lipofuscin is a yellowish brown fluorescent pigment which is sequestered within cytoplasmic granules during aging. To examine the contribution of protein oxidation to lipofuscin accumulation, we performed immunohistochemical detection of dityrosine, which is considered one of the specific markers for protein oxidation, in lipofuscin in the aged human brain using an antibody specific to dityrosine. By characterization using competitive enzyme-linked immunosorbent assay, the specificity of the antibody to dityrosine was confirmed. None of the other tyrosine-related compounds such as L-tyrosine, 3-nitrotyrosine, 3-chlorotyrosine, or 3,4-dihydroxyphenylalanine cross-reacted with the antibody. The anti-dityrosine antibody reacted with lipofuscin granules in the pyramidal neurons of the aged human brain. The results suggest that protein oxidation by free radicals and/or peroxidases may play an important role in lipofuscin accumulation.

Key words: Dityrosine; Lipofuscin; Aging; Oxidation

1. Introduction

Lipofuscin is a yellowish pigment which is accumulated in the cytosol during aging [1]. The significance and relationship of lipofuscin formation to the aging process have remained ambiguous because the chemical composition of the pigment is heterogeneous and scarcely known. Lipofuscin is thought to be an end product of molecular damage to cell organelles by oxygen free radicals [1,2]. The lipid peroxidation products, such as malondialdehyde, have been considered a precursor for lipofuscin pigments [1–5]. The relationship between vitamin E deficiency and lipofuscin formation [6] suggests the occurrence of oxidative stress being the process of pigment generation. However, direct evidence for the contribution of lipid peroxidation to lipofuscin accumulation has not been shown. The involvement of proteins in lipofuscin formation has been reported. The specific storage of subunit c of mitochondrial ATP synthase in lysosomes of neuronal ceroid lipofuscinosis has been reported [7]. In addition, specific modification of lysine residues in the protein was also observed [8]. However, the participation of protein oxidation in lipofuscin formation has not been confirmed.

Some amino acids are susceptible to oxidation and are converted to modified amino acids [9]. Protein-bound carbonyl, one of the specific markers of oxidation, was detected in brains of Parkinson [10] and Alzheimer [11] patients using 2,4-dinitrophenylhydrazine. However, the carbonyl moieties are derived not only from oxidation but also from glycation or lipid decomposition product modification [12,13]. In this way, specific marker(s) is (are) needed for the evaluation of protein oxidation.

The oxidation of tyrosine generates a tyrosyl radical, and *o*,*o*′-dityrosine is then formed by the reaction of two tyrosyl radicals. Dityrosine has been found in the hydrolysates of several structural proteins [14,15] and insoluble proteins from human cataractous lenses [16,17]. Dityrosine is considered to be an endogenous marker from the selective proteolysis of oxidatively modified red blood cell hemoglobin by proteasome [18]. Dityrosine is formed by active oxygen species [19,20], enzymatic reactions [21,22] and UV irradiation [23]. During lipid peroxidation, the formation of dityrosine was also observed [24].

In this report, the method for preparation of anti-dityrosine antibody was demonstrated at first. Using the obtained antibody, we proved the accumulation of dityrosine-like immunoreactivity in lipofuscin granules in the human brain. The antibody to dityrosine may become a good tool for the estimation of localization of dityrosine in oxidative stress-related diseases.

2. Materials and methods

2.1. Materials

L-Tyrosine was purchased from Nacarai Tesque (Kyoto, Japan). Horseradish peroxidase, lipid-free BSA, peroxidase-conjugated anti-rabbit IgG antibody, alkaline phosphatase conjugated anti-rabbit IgG antibody, 3,4-dihydroxyphenylalanine (DOPA), *o*-phosphotyrosine, 3-nitrotyrosine, 3-chlorotyrosine, 3-aminotyrosine, Thr-Tyr-Ser, *Arthromyces* peroxidase, and catalase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (30%) was obtained from Mitsubishi Gas Co. (Tokyo, Japan). Keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Pierce (Rockford, IL, USA). L-Phenylalanine was obtained from Wako Pure Industries (Osaka, Japan).

2.2. Synthesis of dityrosine

Dityrosine was enzymatically prepared using horseradish peroxidase, L-tyrosine, and hydrogen peroxide [23]. The purified dityrosine

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Abbreviations: MDA, malondialdehyde; DOPA, 3,4-dihydroxyphenylalanine; KLH, keyhole limpet hemocyanin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; sulfo-NHS, *N*-hydroxysulfosuccinimide; HPLC, high-performance liquid chromatography; FAB-MS, fast-atom bombardment mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; HNE, 4-hydroxy-2-nonenal; GC-MS, gas chromatography-mass spectrometry

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was identified on the basis of FAB(+)-MS, LC-MS (electrospray ionization +), and ^{1}H-NMR [21,22].

The dimer of Thr-Tyr-Ser by dityrosine linkage was enzymatically prepared using *Arthromyces* peroxidase as already described [25] with some modifications. Briefly, Thr-Tyr-Ser (1 mM) was incubated in 0.25 M borate buffer (pH 8.4) in the presence of 1 mM H_2O_2 and 0.1 mg/ml *Arthromyces* peroxidase at 39°C for 10 min. The reaction was terminated by the addition of 2 mM reduced glutathione and the mixture then kept on ice. The solution (2.5 ml) was applied to a PD-10 column (Pharmacia) which was equilibrated with water. The eluent containing fluorescent compounds was collected and then purified by reversed phase HPLC on a Develosil ODS-HG-5 column (8×250 mm). The elution was done at a flow rate of 2.0 ml/min using 0.1% acetic acid/methanol (14/1) as the solvent. The products were detected using a UV detector (UV 274 nm) and a fluorescent detector (excitation 300 nm, emission 400 nm). The major fluorescent product was collected and concentrated. The dimer formation was confirmed by LC-MS. The presence of dityrosine as one of the components of the dimer was proved by acid hydrolysis with an amino acid analyzer (JLC-500, JEOL) and HPLC.

2.3. Preparation of dityrosine-conjugated proteins

For immunization, dityrosine was conjugated with KLH using EDC as the coupling agent and sulfo-NHS as the enhancer. The protein (7 mg), dityrosine (5.4 mg), EDC (19 mg), and sulfo-NHS (1.8 mg) were dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.4) and reacted for 4 h at room temperature and then overnight at 4°C. The reaction mixture was dialyzed against PBS at 4°C for 3 days. As a coating agent for ELISA, dityrosine was also conjugated with lipid-free BSA using a procedure similar to that already described above. For the purpose of the absorption experiments for immunohistochemistry, dityrosine was conjugated to BSA by glutaraldehyde. Briefly, dityrosine (3.6 mg) and BSA (5 mg) were dissolved in 1.8 ml of 0.1 M phosphate buffer (pH 7.4). Glutaraldehyde (20% solution, 200 μl) was added with stirring. The solution was incubated overnight at room temperature, and then dialyzed against distilled water for 3 days at 4°C.

2.4. Immunization and affinity purification

The dityrosine-conjugated KLH (500 μl, 1 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant to a final concentration of 0.5 mg/ml, and the solution was then intramuscularly injected into a New Zealand White rabbit. After 4 weeks, 500 μl of the modified KLH was emulsified with an equal volume of incomplete adjuvant (0.5 mg/ml), and the emulsified solution was then injected as a booster every 2 weeks until an adequate antibody generation was achieved. The obtained antiserum was purified by an affinity column, which was conjugated with dityrosine on gels. The preparation of the affinity column was performed using Hi-Trap NHS (Pharmacia) according to the manufacturer's recommendation. Briefly, 1 ml of dityrosine solution (1 mg/ml in carbonate buffer, pH 8.3) was loaded on the activated gels (1 ml cartridge) and reacted for 1 h at room temperature. The unbound dityrosine was washed, and the column was then used for the affinity purification. The conjugation of dityrosine to the column was evaluated from the comparison of the original dityrosine solution with the amounts of unbound dityrosine, which was determined by reversed-phase HPLC as described below. The conjugation efficiency was about 61%. The affinity-purified antibody was obtained as follows. The antiserum was loaded on the column and reacted for 1 h at room temperature. The column was washed with water and the antibody was then eluted with 0.1 M Gly-HCl (pH 2.5). The eluate was immediately neutralized with 1 M Tris-HCl (pH 8.0). The antibody solution was divided and stored at −70°C until use.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described [26] with some modifications. Briefly, for non-competitive indirect ELISA, a 50-μl sample of dityrosine-conjugated BSA (0.01 mg/ml), which was prepared using carbodiimide as described above, was added to a microtiter plate and kept 4°C overnight. After washing, the uncovered well was blocked, and serum (1/3000) in PBS containing 0.5% BSA was then added and further incubated for 2 h at 37°C. The binding of the antibody on the coated well was evaluated by adding peroxidase-labeled anti-rabbit IgG antibody. The color development was performed as described [26]. For competitive indirect ELISA, 50 μl of dityrosine-conjugated BSA (0.01 mg/ml) was coated onto a microtiter plate at 4°C overnight. On the other hand, 50 μl of sample (in PBS) and 50 μl of affinity-purified antibody (0.5 μg/ml in PBS containing 1% BSA) were mixed in an Eppendorf tube and further reacted at 4°C for overnight. The microtiter plate was washed and blocked. 90 μl of the sample solution was added to the well and then incubated for 2 h at 37°C. The binding of the antibody on the coated dityrosine-conjugated BSA was evaluated as already described [26]. The result was expressed as B/B_0, where B is the amount of antibody bound in the presence and B_0 the amount in the absence of the competitor. Each point represents the mean of duplicate determinations.

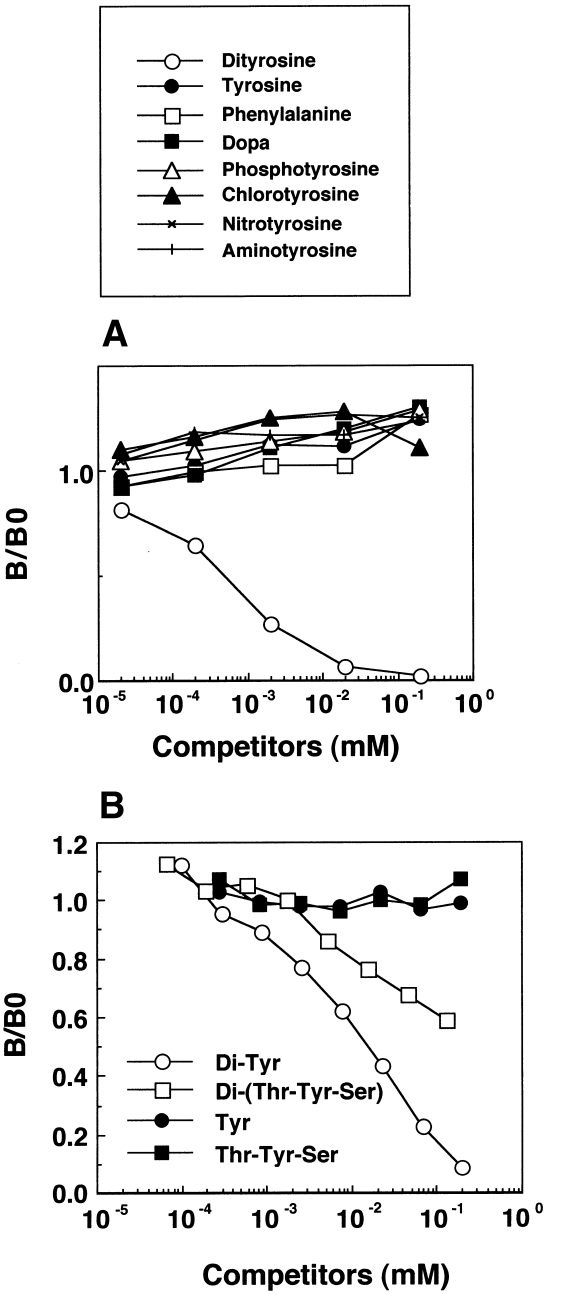

Fig. 1. Cross-reactivities of dityrosine and its related compounds with the antibody (A). The immunoreactivity was estimated by indirect competitive ELISA using dityrosine-conjugated BSA as a coating agent. Comparison of the immunoreactivity between peptidyl dityrosine (Di-(Thr-Tyr-Ser)) and free dityrosine (B). The peptidyl dityrosine was prepared from Thr-Tyr-Ser by treatment with *Arthromyces* peroxidase and hydrogen peroxide.

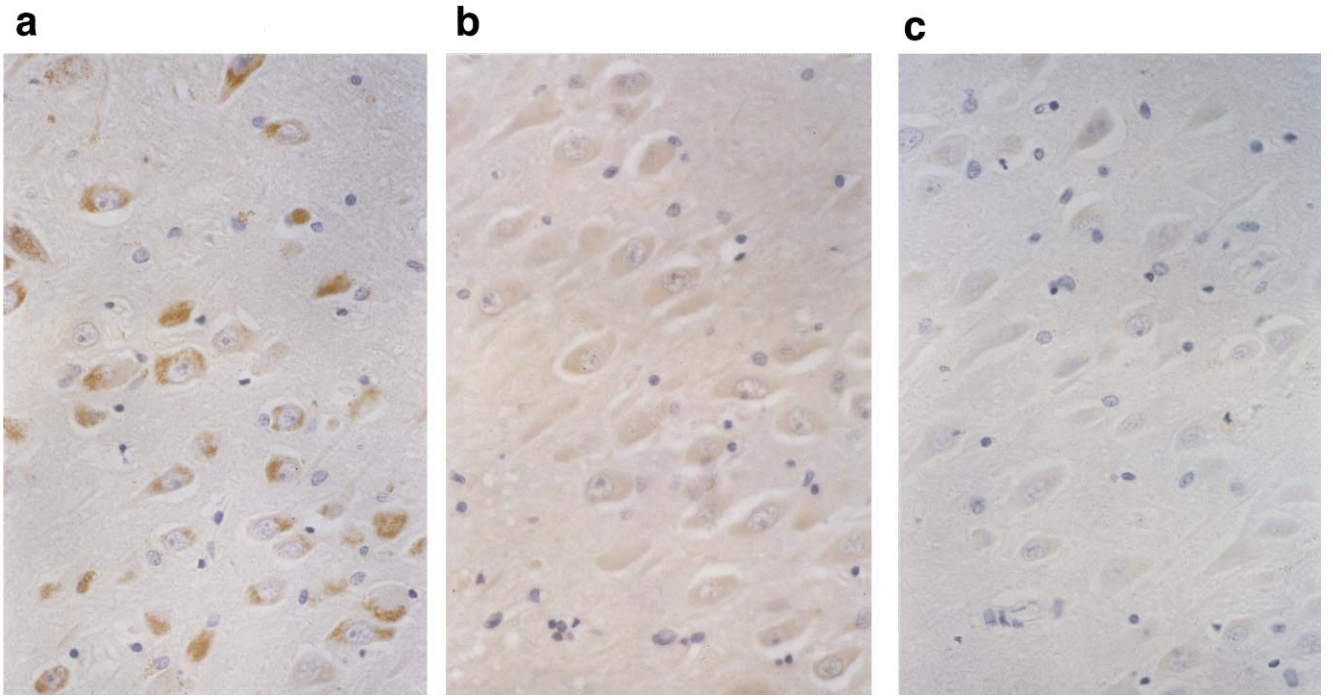

Fig. 2. Immunohistochemical detection of dityrosine in lipofuscin granules in pyramidal hippocampal neurons. The tissue samples were incubated with (a) anti-dityrosine antibody (diluted 1:50 with BSA), anti-dityrosine antibody preabsorbed with (b) dityrosine-BSA, or (c) bovine serum albumin alone, followed by the treatment of peroxidase-labeled anti-rabbit IgG. The lipofuscin granules were positively stained (a).

2.6. Chemical detection of dityrosine

The quantification of dityrosine in the conjugated proteins was performed by HPLC as previously described [23]. The acid hydrolysates of the modified proteins were dissolved in 0.01 M HCl and then applied to the Develosil ODS-5 column (4.6×250 mm) equilibrated with 0.5% acetic acid/methanol (29/1). The elution was performed at a flow rate of 0.8 ml/min and monitored by the fluorescence of dityrosine (excitation 300 nm; emission 400 nm). The amount of dityrosine was calculated from a standard curve using synthetic dityrosine. The concentration of synthetic dityrosine was determined based on its molar coefficient at 284 nm, 5400 [24]. The identification of the dimer from Thr-Tyr-Ser was performed by LC-MS. The sample was applied to a liquid chromatograph on a Develosil ODS-HG-5 column (4.6×250 mm), which was connected with a mass spectrometer (PLATFORM II, VG Biotech). The separation was isocratically performed. The solvent was 0.1% acetic acid/acetonitrile (1/1). The electrospray ionization (positive) mode was used for the detection.

2.7. Immunohistochemistry

Samples from three patients (without history of neurological diseases) were analyzed in the present study. The age, sex, and cause of death were as follows: 68, male, dissecting aneurysm; 65, female, myocardial infarction; 74, male, lung cancer. Brains were obtained at autopsy and preserved fixed in formalin and embedded in paraffin. Immunohistochemical examination was performed by the streptavidin-biotin complex method according to the manufacturer's instructions (Dako kit, Dako, Kyoto, Japan). After deparaffinization with xylene and ethanol, the sample was treated with 0.1% trypsin (Dako) for 15 min at 37°C. The sample was incubated with 3% hydrogen peroxide for 5 min, bovine serum for 5 min at room temperature and then rabbit anti-dityrosine antibody (diluted 1:50 by bovine serum albumin), the antibody preabsorbed with dityrosine-conjugated bovine serum albumin (BSA), or BSA alone as a negative control for 1 h at 37°C. The antigen-antibody complex was then incubated with biotin-labeled sheep anti-rabbit IgG serum followed by peroxidase labeled streptavidin for 20 min. Detection was done by staining the sample with 3% 3,3-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. After the immunostaining, the sample was stained with hematoxylin.

3. Results and discussion

3.1. Preparation of a polyclonal antibody to dityrosine

Dityrosine was prepared by horseradish peroxidase and L-tyrosine in the presence of hydrogen peroxide as described in Section 2. The obtained dityrosine was chromatographically pure, and the mass and ^{1}H-NMR spectra were identical to the previous results [21,22]. The conjugation of dityrosine with KLH was performed by carbodiimide as described in Section 2. The binding of dityrosine to KLH was 20 μmol dityrosine/g protein. The modified KLH was injected into a rabbit intramuscularly.

The generation of antibody against dityrosine was examined by ELISA. The time-dependent formation of immunoreactivity on coated dityrosine-conjugated BSA (26.5 μmol dityrosine/g protein) prepared by carbodiimide was observed (data not shown). The antiserum was then collected, and the antibody was purified using a dityrosine-conjugated column.

3.2. Characterization of anti-dityrosine antibody

Dityrosine was also conjugated to BSA with glutaraldehyde, and the cross-reactivity of the conjugates with the affinity-purified antibody was examined. The antibody recognized the glutaraldehyde-conjugated BSA, suggesting that the antibody reacted with dityrosine but not with the coupling agent-derived compounds. Furthermore, we investigated the binding of various low molecular weight compounds (dityrosine, L-tyrosine, L-phenylalanine, 3-nitrotyrosine, 3-chlorotyrosine, 3-aminotyrosine, *o*-phosphotyrosine, and 3,4-dihydroxyphenylalanine) using competitive indirect ELISA. Fig. 1A shows that the obtained antibody specifically recognized free dityrosine but not other tyrosine-related compounds. As shown in Fig. 1B, the antibody also recognized the peptidyl dityro-

sine, a dimer of Thr-Tyr-Ser, but the reactivity was weak compared to the 'free' dityrosine.

3.3. Identification of dityrosine in lipofuscin in brain

Using the antibody specific to dityrosine, the immunohistochemical detection in the human brain hippocampus was examined. As shown in Fig. 2a, immunopositive materials against the affinity-purified anti-dityrosine antibody were observed in the lipofuscin granules of the pyramidal neurons. On the other hand, no positive staining was observed with the omission of the anti-dityrosine antibody (Fig. 2b) and with the anti-dityrosine antibody preabsorbed with dityrosine-conjugated BSA (Fig. 2c). As far as we know, dityrosine could be immunohistochemically detected in lipofuscin pigments of the brain for the first time. The contribution of lipid peroxidation to the lipofuscin formation has been interesting but the effect of oxidized protein on the formation has not been reported. The fluorescence wavelength of lipofuscin is different from that of dityrosine [1]. Dityrosine may not contribute to the characteristic 'fluorescence' of lipofuscin. However, dityrosine was accumulated in lipofuscin pigments, suggesting that oxidative protein modifications would contribute to the pigment formation at least in part, whereas the in vivo oxidants of tyrosine residues remain unknown; peroxidases such as myeloperoxidase [27–30] as well as free radicals [19,20] may contribute to the formation of dityrosine in the brain. It has been reported that endogenous dityrosine in human cerebrospinal fluid was detected [31]. Tyrosyl radicals, the precursor for dityrosine, can cause lipid peroxidation [32]. Both tyrosyl radicals and lipid decomposition products probably participate in the lipofuscin formation. More detailed studies are needed for elucidation of the mechanism of dityrosine formation in lipofuscin.

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