

Immunohistochemical Localization of Advanced Glycation End Products, Pentosidine, and Carboxymethyllysine in Lipofuscin Pigments of Alzheimer's Disease and Aged Neurons

Katsunori Horie,* Toshio Miyata,† Takeshi Yasuda,‡ Akinori Takeda,‡ Yoshinari Yasuda,* Kenji Maeda,* Gen Sobue,‡ and Kiyoshi Kurokawa†¹

*Department of Internal Medicine and ‡Department of Neurology, Nagoya University School of Medicine, Nagoya 466; and †Institute of Medical Sciences and Department of Medicine, Tokai University School of Medicine, Isehara 259-11, Japan

Received June 12, 1997

Lipofuscins are intracellular fluorescent pigments accumulating in the central nervous system (CNS) with aging and degenerative processes such as Alzheimer's disease (AD). Although they are thought to be lipid peroxidation products derived from malondialdehyde, their biogenesis remains controversial. We further characterize the chemical nature of lipofuscins in brain tissues from AD patients and normal aged subjects. Advanced glycation end products (AGEs), pentosidine and carboxymethyllysine (CML), were identified by appropriate specific antibodies. They have physicochemical properties similar to those of lipofuscin and also increase with aging. Pentosidine and CML were identified in the neuronal perikarya and the extraneuroperikaryal deposits of both the AD and aged brain. Pentosidine, but not CML, was present in the fiber-like structure within the neuropil and the core of classical senile plaque. In the brain of young subjects without CNS disease, pentosidine and CML staining was faint. Pentosidine and CML co-localized with lipofuscin pigments in the neuronal perikarya of both the AD and aged brain. We demonstrate for the first time that lipofuscin is constituted not only of lipid peroxidation products but also from glycation products which may be the origin of fluorescent pigments. Lipofuscins should

thus be considered as fluorescent pigments generated by lipid- and sugar-derived Schiff base-protein polymers. © 1997 Academic Press

Lipofuscins, intracellular fluorescent pigments, accumulate in the central nervous system with aging and degenerative processes such as Alzheimer's disease (AD)(1). The pathophysiological significance and the origin of lipofuscins are not well understood. Several histochemical and biochemical studies suggest that li-

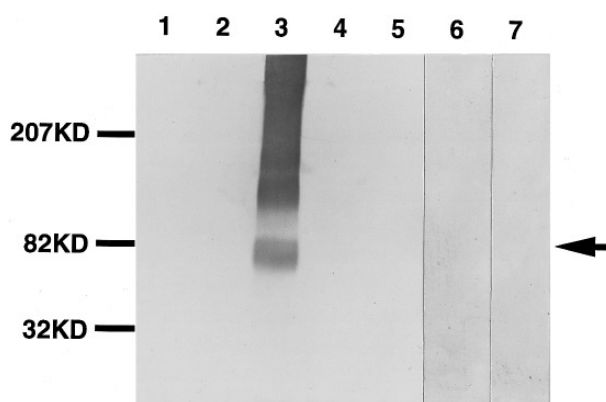


FIG. 1. Characterization of anti-pentosidine antibody. The samples were resolved by 15% SDS-PAGE, followed by immunoblotting using anti-pentosidine rabbit IgG. Lane 1, normal BSA; lane 2, Amadori-BSA; lanes 3, 6, and 7, pentosidine-BSA; lane 4, CML-BSA; lane 5, pyrraline-BSA. Non-immune rabbit IgG was used as a negative control (lane 6), or the antibody preincubated with free pentosidine was used for a competition experiment (lane 7). The position of BSA (arrows) and migration of standard proteins are indicated.

¹ Corresponding author. Institute of Medical Sciences and Department of Medicine, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-11, Japan. Fax: 81-463-93-1130. E-mail: kurokawa@is.icc.u-tokai.ac.jp.

Abbreviations used: CNS, central nervous system; AD, Alzheimer's disease; AGEs, advanced glycation end products; CML, N ϵ -(carboxymethyl)lysine; KLH, keyhole limpet hemocyanin; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin.

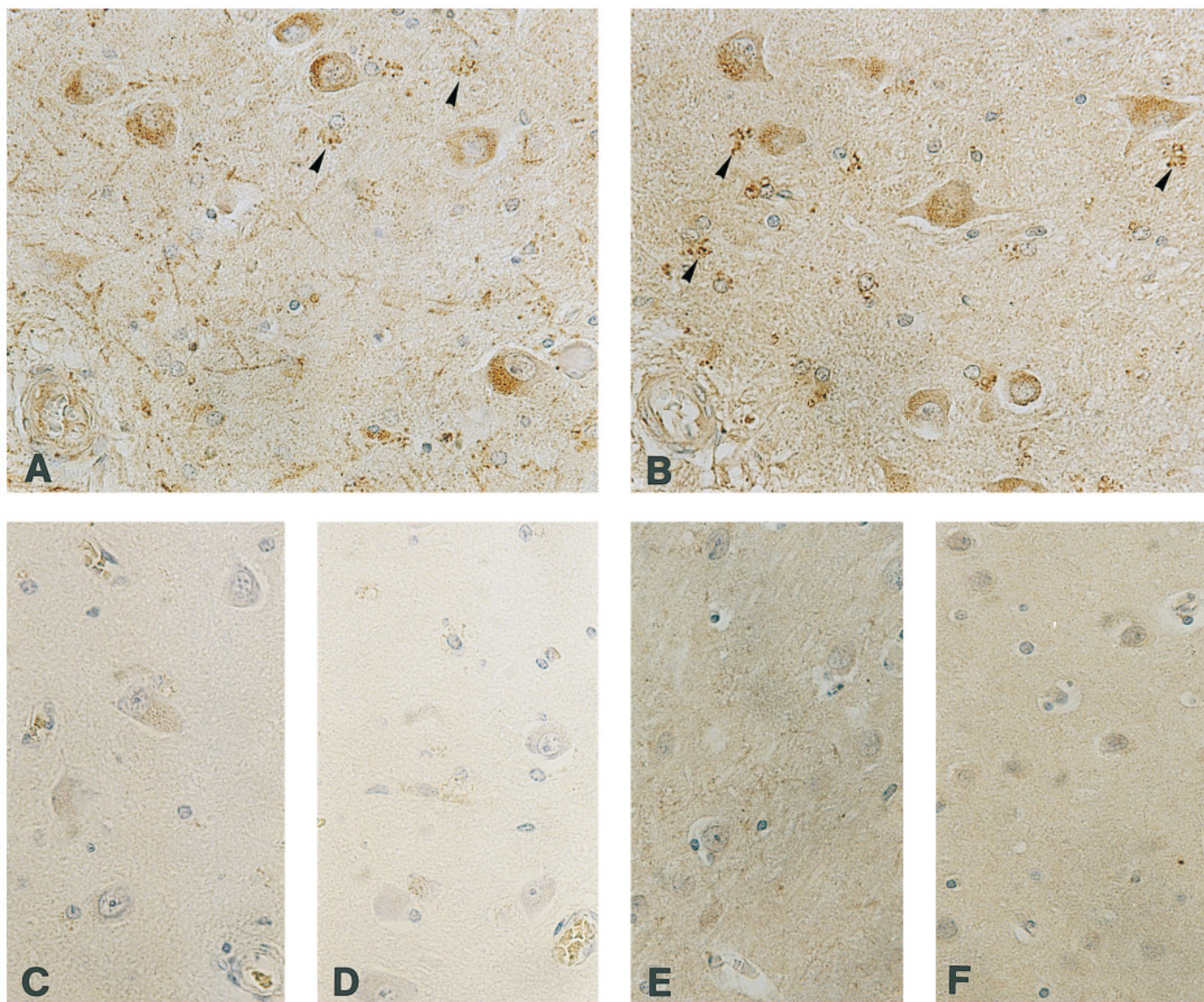


FIG. 2. Detection of pentosidine and CML in the AD and the young brain. Brain tissue sections obtained from a 81-year-old AD patient (A-D) or a 17-year-old man (E and F) were stained with anti-pentosidine antibody (A, C and E) and 6D12 (B, D and F). The anti-pentosidine antibody and 6D12 preincubated with an excess of free pentosidine and AGE-BSA, respectively, were used for the competition experiment (C and D). The nuclei were counterstained with Meyer's hematoxylin. Original magnification: A-F, $\times 400$. Pentosidine and CML were found in the cytoplasm of neurons and the extra-neuropil deposits (arrowheads) in the neuropil of the AD brain (A and B), but not in young neurons (E and F). Pentosidine was also found in the fiber-like structure within the neuropil (A).

pofuscins are pigments generated during oxidative processes of poly-unsaturated fatty acid components of membrane lipids (2). It has been claimed that lipofuscins are derived from malondialdehyde (2). Consistent with this hypothesis, Monji *et al.* (3) reported the accumulation of lipofuscins in the brain of mice deficient of vitamin E, an antioxidant, free radical scavenger protecting against lipid peroxidation. By contrast, d'Ischia *et al.* (4) demonstrated that the reaction of malondialdehyde with amines *per se* contributes minimally to the development of fluorescent pigments. They suggest

that the chromo- and fluoro-phoric properties of lipofuscins are generated by other, as yet, unknown mechanisms different from a mere condensation with malondialdehyde. The chemical composition of lipofuscin does not fit the lipid peroxidation theory: protein 30 %, cholesterol 6.3 %, phospholipid 1.3 %, and carbohydrate up to 36 % (2). The role of malondialdehyde in the biogenesis of related fluorescent pigments remains thus controversial.

Glucose reacts non-enzymatically with protein amino group, known as the Maillard reaction, to reversibly

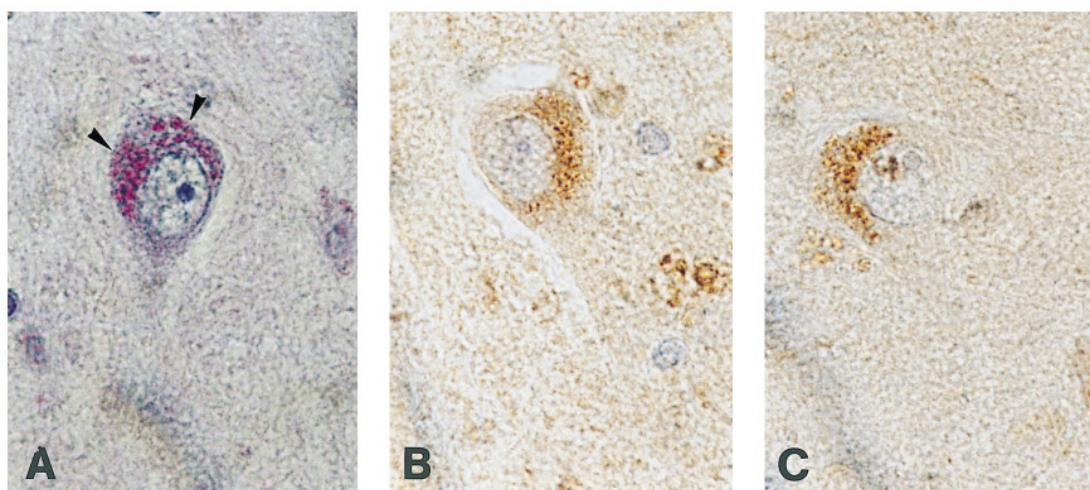


FIG. 3. Detection of AGE structures, pentosidine and CML, in the intraneuronal lipofuscin. Brain tissue sections obtained from a 81-year old AD patient was stained with periodic acid Schiff reaction (A), anti-pentosidine antibody (B), and 6D12 (C). Original magnification: $\times 1000$. The lipofuscin pigments (arrowheads in A) in the neuron of the CA4 region coincided with pentosidine and CML deposits.

form Schiff base, and upon rearrangement, convert into more stable Amadori product (5, 6). Through a series of chemical rearrangements, dehydration, and fragmentation reactions, Amadori products are further converted, over months, into the pigmented and fluorescent adducts, called the advanced glycation end products (AGEs) (5, 6). This process is so slow that it affects primarily proteins with a slow turnover. It is not surprising to find that AGE levels slowly increase with age in a variety of collagenous structures (7, 8). AGEs constitute a heterogeneous class of structure such as pentosidine (9) and *N*-(carboxymethyl)lysine (CML) (10).

The physicochemical properties of AGEs such as pigmentation and fluorescence and the increase of AGEs levels with aging suggest a potential link between AGEs and the genesis of fluorescent lipofuscin pigments. To test this hypothesis, brain tissues from patients with AD and from the aged subjects were examined to detect pentosidine or CML with specific antibodies.

MATERIALS AND METHODS

Specimens. Brain tissues were obtained at autopsy from twenty patients. Ten had clinicopathologically confirmed Alzheimer's disease (age range 80-87), eight were over seventy years old (age range 71-87) without dementia, and two young subjects (15 and 17 years old) had suffered from progressive muscular dystrophy and epilepsy. The hippocampus and parahippocampus were excised, fixed in 10 % buffered-formalin and embedded in paraffin.

Antibodies. Anti-pentosidine rabbit antibody was produced by immunization of rabbit with keyhole limpet hemocyanin (KLH) modified with synthesized pentosidine. Pentosidine was synthesized by a method adapted from Grandhee and Monnier (11). A suspension of 0.01 mol of *N*^ε-*t*-butoxycarbonyl-L-lysine and 0.08 mol of D-ribose in 100 ml of methanol was stirred for 3 h at 30 °C. Methanol was evaporated under reduced pressure resulting in a dark brown syrup.

The residue was purified by column chromatography on Dowex 50 W \times 2 (Aldrich, Milwaukee, WI) using a linear gradient from 0.2 M pyridine acetate (pH 3.1) to 2 M pyridine acetate (pH 5.0). The main fraction (*N*^ε-*t*-butoxycarbonyl-*N*-(1-deoxy-D-ribulos-1-yl)-L-lysine) was collected, concentrated *in vacuo* and lyophilized to give a partially purified product (4.15 g). The product thus obtained (0.01 mol) and *N*^ε-*t*-butoxycarbonyl-L-arginine (0.042 mol) were dissolved in 100 ml of sodium phosphate buffer (pH 9.4). The pH of the solution was adjusted to 11-12 with 1 N NaOH. After stirring for 20 h at room temperature, the solution was acidified to pH 2 with 6 N HCl and concentrated *in vacuo* to give an oily residue. The *t*-butoxycarbonyl groups were removed by treatment with 300 ml of trifluoroacetic acid for 1 h at room temperature. After removal of excess trifluoroacetic acid *in vacuo*, the residue was purified on high performance liquid chromatography (HPLC) using a reverse-phase column (YMC-Pack ODS: YMC Corp., Kyoto, Japan) to give a homogeneous product (70 mg). The identity of the final product was confirmed as pentosidine by nuclear magnetic resonance and fast atom bombardment-mass spectrometry.

To prepare pentosidine-modified KLH, synthesized pentosidine was conjugated with KLH by our previous method (12). Briefly, 10 mg of KLH (Pierce, Rockford, IL) was incubated with 7.5 mmol of pentosidine and 0.1 mmol of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (Pierce) in the presence of 2.2 mg of *N*-hydroxysulfosuccinimide (Pierce) in 3 ml of phosphate buffered saline (PBS) for 4 h at room temperature, and then overnight at 4°C. Pentosidine incorporation into KLH was 45.3 pmol/mg, as determined by HPLC assay (12).

Pentosidine-modified KLH was then used to immunize rabbits by standard methods, and IgG from immune serum was purified on affinity protein A agarose using a kit (Affi-Gel Protein A MAPS II Kit: Bio-Rad, Richmond, CA). Thus-obtained IgG fraction was further affinity-purified by adsorbing to CNBr-activated Sepharose 4B column (Pharmacia, Uppsala, Sweden) with immobilized bovine serum albumin (BSA) modified with pentosidine (5 mg/ml gel), washing extensively with 20 mM sodium phosphate buffer (pH 7.4), and eluting bound immune IgG with the same buffer containing 3 M potassium thiocyanate (pH 7.4). IgG was then dialyzed against 20 mM sodium phosphate buffer (pH 7.4).

Anti-AGE mouse monoclonal IgG (6D12)(13), the epitope-structure of which was recently identified as CML (14, 15), was also purchased from Wako Pure Chemicals (Osaka, Japan).

Immunoblot analysis. BSA modified with the early Maillard Amadori products (Amadori-BSA) was prepared by incubating 500 mg of BSA (essentially fatty acid-free grade; Sigma, St. Louis, MO) with 0.1 M D-glucose (Wako Pure Chemicals) at 37 °C for 10 days in 500 ml of 0.1 M phosphate buffer (pH 7.4). The level of Amadori products (fructoselysine) in the glycated BSA after a 10-day incubation was 0.44 mol of fructoselysine *per* mol of BSA, as determined by a colorimetric assay (16) using a kit (Fructosamine Test Roche-II; Nihon Roche, Tokyo, Japan). To prepare BSA linked with CML (CML-BSA) or pentosidine (pentosidine-BSA), synthesized CML (15) or pentosidine were conjugated with BSA as described above. BSA linked with pyralline (pyralline-BSA) was kindly provided from Dr. Satoshi Miyata.

The samples thus-prepared (10 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15 % gradient acrylamide gel. After electrophoretic transfer to a polyvinylidene difluoride membrane (Bio-Rad), the membrane was incubated with 3 % skim milk overnight, and reacted for 1 h at room temperature with anti-pentosidine rabbit IgG (10 µg/ml) in the buffer containing 25 mM Tris-HCl (pH 7.5) and 0.5 M NaCl, followed by washings with the same buffer containing 0.1 % Tween-20. The membrane was incubated with 1:5,000 diluted goat anti-rabbit IgG conjugated with alkaline phosphatase (Organon Teknica, Durham, USA) for 1 h at room temperature, washed, and then reacted with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium solution (GIBCO, Gaithersburg, MD). For an inhibition test, anti-pentosidine antibody preincubated with an excess of free pentosidine for 2 h at 37 °C was used. Non-immune rabbit IgG was used as a negative control.

Immunohistochemistry. Brain tissue sections cut at 5 µm were mounted on slides coated with 3-aminopropyltriethoxy silane, deparaffined, rehydrated in the distilled water, and heated in a microwave oven. The sections were washed with PBS containing 0.05 % Tween, incubated in 0.3 % H₂O₂ in methanol for 30 min, and further blocked with 1 % BSA in BPS for 2 h. They were again incubated with either 6D12 (0.5 µg/ml) or anti-pentosidine rabbit IgG (10 µg/ml) in humid chambers for 2 h at room temperature. Detection was performed using an ABC staining kit (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). Competition experiments to confirm the specificity of immunostaining was also done with 6D12 or anti-pentosidine rabbit IgG, which was preincubated with an excess of CML-BSA or free pentosidine, respectively, for 2 h at 37 °C. Non-immune mouse or rabbit IgG was used as a negative control. Lipofuscin pigments were identified in serial brain tissue sections stained with periodic acid schiff (PAS), periodic acid-methenamine-silver (PAM), and hematoxylin-eosin (HE).

RESULTS

Characterization of Anti-pentosidine Antibody

On the immunoblot analysis (Fig. 1), the anti-pentosidine rabbit IgG reacted with pentosidine-BSA (lane 3) and pentosidine-KLH (data not shown). No immunoreaction was observed with either BSA (lane 1), Amadori-BSA (lane 2), CML-BSA (lane 4), or pyralline-BSA (lane 5). The immunoreactivity of the anti-pentosidine antibody was completely abolished after preincubation with an excess of synthesized pentosidine (lane 6), indicating a high specificity of the immunoreaction. No immunoreaction was detected with non-immune rabbit IgG (lane 7). These findings indicate that this antibody specifically recognizes pentosidine.

The present immunohistochemical study was per-

formed with this anti-pentosidine antibody and with 6D12 which specifically recognizes CML. Both pentosidine and CML will be referred to in the text as AGEs.

Detection of AGE Structures, Pentosidine and CML, in the AD and Aged Brain

In the AD brain, pentosidine and CML were identified in the perikarya of hippocampal pyramidal neurons (Figs. 2A, B). AGE-positive neurons were more prominent in the parahippocampal gyrus and CA3, 4 areas than in the CA1 area. Both pentosidine and CML were present in the extra-neuroperikaryal deposits in the neuropil of the parahippocampal and CA4 areas. Pentosidine was also found in the fiber-like structure within the neuropil (Fig. 2A) and the core of classical senile plaque, whereas CML was not detectable in these structures. The CML staining pattern was in good agreement with that previously reported by us (17) and other research groups (18). No immunoreaction was observed in the AD brain with non-immune mouse or rabbit IgG, with anti-pentosidine antibody or 6D12 preincubated with an excess of free pentosidine or CML-BSA, respectively (Figs. 2C, D).

In the brain of elderly subjects, pentosidine and CML were detected in the neuronal perikarya and in the extraneuron, but the number of extraneuronal AGE deposits was lower than in the AD brain (data not shown).

In the brain of young subjects, only faint deposits of pentosidine and CML were found in the perikarya and extraneuron (Figs. 2E, F).

AGE Structures and Intraneuronal Lipofuscin Deposits

The topographical relationship between AGE deposits and lipofuscin in the neuronal perikarya was then evaluated. The topography of intraneuronal pentosidine and CML deposits was identical with that of lipofuscin pigments identified by PAS (Fig. 3A), PAM and HE (data not shown) staining in both the AD (Fig. 3B, C) and aged brain.

DISCUSSION

AGEs are pigmented and fluorescent adducts formed by a non-enzymatic reaction between sugar and long-lived protein. In the present study, we demonstrate that AGE development is closely related to the biogenesis of fluorescent lipofuscin pigments in the brain. Pentosidine and CML, both AGE structures, were present in the neuronal perikarya and co-localized with lipofuscin. Glucose-derived Schiff base adducts are thus chemical components of lipofuscin, which now appears generated by lipid- and sugar-derived Schiff base-protein polymers. Considering the recent finding by d'Ischia *et*

al. (4) that malondialdehyde is a possible contributor, but not the primary determinant of fluorescent pigment formation, the generation of chromo- and fluorophoric properties of lipofuscin may be derived from AGEs rather than malondialdehyde. Our present finding is in good agreement with the previous report by others that carbohydrate is a significant component of lipofuscin as compared to lipid components (2).

Pentosidine, but not CML, was observed in the fiber-like structure of the neuropil and senile plaque. One possible explanation for this discrepancy is the difference of the precursor for these AGE structures. Although glucose is thought to be a major precursor for AGEs, recent studies have demonstrated that pentosidine and CML can also be formed *in vitro* by reactions of protein with ascorbate, ribose, and other carbohydrate sources (11, 19, 20). Therefore, *in vivo*, pentosidine and CML may have different precursors. Interestingly, from the pathological perspective, pentosidine might contribute to the extraneuronal fiber-like structure and senile plaque due to its characteristic property as a potent protein cross-linker (9), a property not present in CML. The possible involvement of pentosidine in amyloid fibril formation has been shown in dialysis-related amyloidosis (12) and Alzheimer's disease (21).

Several lines of evidence have demonstrated that AGEs may play a role in the development of diabetic (8, 22, 23) and uremic (24) complications through a variety of mechanisms: the alteration of structure and function of tissue proteins (5) and stimulation of cellular responses, *e.g.*, monocyte chemotaxis (25-27), macrophage secretion of cytokines (26-29), and osteoclast-induced bone resorption (30, 31). At present, however, the pathological significance of AGEs in the central nervous system, especially within the neuron, remains unknown. Further study will be required to address this interesting issue.

Although the pathological significance of AGEs in the brain remains unclear, recent studies have clearly demonstrated that the formation of pentosidine and CML is closely linked to oxidative processes (32-35). Pentosidine and CML are, therefore, referred to as glycoxidation products (32) and may serve as markers of oxidative stress damage, just as lipid peroxidation products. Within this context, it is noteworthy that pentosidine and CML co-localized with lipofuscin, a significant constituent of which is thought to be lipid peroxidation products such as malondialdehyde. Previous study by Monji *et al.* (3) demonstrating the accumulation of lipofuscin in the brain of mice deficient of antioxidant vitamin E, also supports this notion. The relationship between AGE accumulation and oxidative stress is currently investigated in our laboratory.

Finally, it is noteworthy that AGEs accumulate with aging in the brain just as they do in a variety of other tissues: both pentosidine and CML were initially almost

absent in the young brain, present in the aged brain, and more accentuated in the AD brain.

ACKNOWLEDGMENTS

We thank Professor Charles van Ypersele de Strihou for critically reviewing the manuscript. This study was supported by a grant from Research for the Future Program of the Japan Society for the Promotion of Science (96L00303).

REFERENCES

1. Dowson, J. H. (1982) *Brit. J. Psych.* **140**, 142-148.
2. Jolly, R. D., Dalefield, R. R., and Palmer, D. N. (1993) *J. Inher. Dis.* **16**, 280-283.
3. Monji, A., Morimoto, N., Okuyama, I., Yamashita, N., and Tashiro, N. (1994) *Brain Res.* **634**, 62-68.
4. d'Ischia, M., Costantini, C., and Protta, G. (1996) *Biochim. Biophys. Acta* **1290**, 319-326.
5. Brownlee, M., Cerami, A., and Vlassara, H. (1988) *N. J. Med.* **318**, 1315-1321.
6. Baynes, J. W., and Monnier, V. M. (1989) *Prog. Clin. Biol. Res.* **304**, 1-410.
7. Dyer, D. G., Duun, J. A., Thorpe, S. R., Bailie, K. E., Lyons, T. J., MaCane, D. R., and Baynes, J. W. (1993) *J. Clin. Invest.* **91**, 2463-2469.
8. Beisswenger, P. J., Moore, L. L., and Brink-Johnsen, T. (1993) *J. Clin. Invest.* **92**, 212-217.
9. Sell, D. R., and Monnier, V. M. (1990) *J. Clin. Invest.* **85**, 380-384.
10. Ahmed, M. U., Thorpe, S. R., and Baynes, J. W. (1986) *J. Biol. Chem.* **261**, 4889-4894.
11. Grandhee, S. K., and Monnier, V. M. (1991) *J. Biol. Chem.* **266**, 11649-11653.
12. Miyata, T., Taneda, S., Kawai, R., Ueda, Y., Horiuchi, S., Hara, M., Maeda, K., and Monnier, V. M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 2353-2358.
13. Horiuchi, S., Araki, N., and Morino, Y. (1991) *J. Biol. Chem.* **266**, 7329-7332.
14. Ikeda, K., Higashi, T., Sano, H., Jinnouchi, Y., Yoshida, M., Araki, T., Ueda, S., and Horiuchi, S. (1996) *Biochemistry* **35**, 8075-8083.
15. Miyata, T., Wada, Y., Cai, Z., Iida, Y., Horie, Yoshinari Yasuda, K., Maeda, K., Kurokawa, K., and van Ypersele de Strihou, C. (1997) *Kidney Int.* **51**, 1170-1181.
16. Miyata, T., Iida, Y., Ueda, Y., Shinzato, T., Seo, H., Monnier, V. M., Maeda, K., and Wada, Y. (1996) *Kidney Int.* **49**, 538-550.
17. Takeda, A., Yasuda, T., Miyata, T., Mizuno, K., Li, M., Yoneyama, S., Horie, K., Maeda, K., and Sobue, G. (1996) *Neurosci. Lett.* **221**, 17-20.
18. Kimura, T., Takamatsu, J., Ikeda, K., Kondo, A., Miyakawa, T., and Horiuchi, S. (1996) *Neurosci. Lett.* **46**, 757-763.
19. Dunn, J. A., Ahmed, M. U., Murtiashaw, M. H., Richardson, J. M., Walla, M. D., Thorpe, S. R., and Baynes, J. W. (1990) *Biochemistry* **29**, 10964-10970.
20. Dyer, D. G., Blackledge, J. A., Thorpe, S. R., and Baynes, J. W. (1991) *J. Biol. Chem.* **266**, 11654-11660.
21. Smith, M. A., Taneda, S., Richey, P. L., Miyata, S., Yan, S. D., Dtern, D., Sayre, L. M., Monnier, V. M., and Perry, G. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 5710-5714.

22. Sell, D. R., Lapolla, A., Odetti, P., Forgarty, J., and Monnier, V. M. (1992) *Diabetes* **41**, 1286–1292.
23. McCance, D. R., Dyer, D. G., Duun, J. A., Bailie, K. E., Thrope, S. R., Baynes, J. W., and Lyons, T. J. (1993) *J. Clin. Invest.* **91**, 2470–2478.
24. Miyata, T., Oda, O., Inagi, R., Iida, Y., Araki, N., Yamada, N., Horiuchi, S., Taniguchi, N., Maeda, K., and Kinoshita, T. (1993) *J. Clin. Invest.* **92**, 1243–1252.
25. Kirstein, M., Brett, J., Radoff, S., Ogawa, S., Stern, D., and Vlassara, H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 9010–9014.
26. Miyata, T., Inagi, R., Iida, Y., Sato, M., Yamada, N., Oda, O., Maeda, K., and Seo, H. (1994) *J. Clin. Invest.* **93**, 521–528.
27. Miyata, T., Hori, O., Zhang, J. H., Yan, S. D., Ferran, L., Iida, Y., and Schmidt, A. M. (1996) *J. Clin. Invest.* **98**, 1088–1094.
28. Vlassara, H., Brownlee, M., Manogue, K. R., Dinarello, C. A., and Pasagian, A. (1998) *Science* **240**, 1546–1548.
29. Iida, Y., Miyata, T., Inagi, R., Sugiyama, S., and Maeda, K. (1994) *Biochem. Biophys. Res. Commun.* **201**, 1235–1241.
30. Miyata, T., Kawai, R., Taketomi, S., and Sprague, S. M. (1996) *Nephrol. Dial. Transplant.* **11**(suppl 5), 54–57.
31. Miyata, T., Notoya, K., Yoshida, K., Horie, K., Maeda, K., and Taketomi, S. (1997) *J. Am. Soc. Nephrol.* **8**, 260–270.
32. Baynes, J. W. (1991) *Diabetes* **40**, 405–412.
33. Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R., and Baynes, J. W. (1995) *Biochemistry* **34**, 3702–3709.
34. Wells-Knecht, M. A., Thorpe, S. R., and Baynes, J. W. (1995) *Biochemistry* **34**, 15134–15141.
35. Glomb, M. A., and Monnier, V. M. (1995) *J. Biol. Chem.* **270**, 10017–10026.