Age- and Stage-dependent Accumulation of Advanced Glycation End Products in Intracellular Deposits in Normal and Alzheimer’s Disease Brains

In this immunohistochemical study, the age- and stage-dependent accumulation of advanced glycation end-products (AGEs) in Alzheimer’s disease (AD) and their relation to the formation of neurofibrillary tangles and neuronal cell death was investigated. For this purpose, the distribution of AGEs in neurons and glia was analyzed in the auditory association area of superior temporal gyrus (Brodmann area 22) of young and old non-demented controls and compared with early- and late-stage AD. A possible co-localization of AGEs with typical hallmarks of AD, such as hyperphosphorylated tau (as a marker for disturbed kinase/phosphatase activity), nNOS (as a marker for nitroxidative stress) and caspase-3 (as a marker of apoptotic cell death), was also investigated. Our results show that the percentage of AGE-positive neurons (and astroglia) increase both with age and, in AD patients, with the progression of the disease (Braak stages). Interestingly, nearly all if those neurons which show diffuse cytosolic AGE immunoreactivity also contain hyperphosphorylated tau, suggesting a link between AGE accumulation and the formation of early neurofibrillary tangles. Many, but not all, neurons show a co-localization of AGEs with other markers of neurodegeneration, such as nNOS and caspase-3.

Keywords: advanced glycation endproducts, Alzheimer’s disease, Braak stages, neurofibrillary tangles, nitric oxide

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disease that affects a growing proportion of the elderly population. The clinical manifestation of this age-related neurodegenerative disease is gradual onset with progressive memory loss and cognitive dysfunction. The histological hallmarks of AD include widespread neuronal cell death and the formation of neurofibrillary tangles (NFTs) and amyloid plaques. NFTs show a hierarchical spreading pattern from the allocortex to isocortical association areas with the early involvement of the entorhinal region, a major relay station between the hippocampus and isocortex (Braak and Braak, 1991). NFTs consist mainly of abnormally phosphorylated tau protein (Braak et al., 1993). Tau can be phosphorylated at many sites and by several kinases, e.g. mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK-3), cyclin-dependent kinase 5 (cdk5) and JNK/p38 (Atzori et al., 2001; Oth et al., 2002; Pci et al., 2002). Nineteen phosphorylated sites have been identified with most localized to the amino- and carboxyl-terminal flanking regions of the microtubule-binding domain (Reynolds et al., 2000). NFTs go through three different stages of development: stage 1, pre-NFT; stage 2, intra-neuronal NFTs; and stage 3, extra-neuronal NFTs, with possibly all present simultaneously in the brain of a patient in one stage of the AD (Augustinack et al., 2002).

Senile plaques (SP) are complicated lesions composed of amyloid peptides, plaque-associated molecules and degenerating neuronal processes (Braak et al., 1989). Diffuse amyloid deposits evolve over time with formation of discrete seeds that eventually become neuritic SP. In response to interaction with amyloid peptides and other pro-inflammatory factors, microglia and astrocytes produce a number of molecules that may be locally toxic to neuronal processes in the vicinity of SP (Lue et al., 2001). This includes cytokines, reactive oxygen and nitrogen intermediates, and proteases (Gasic-Milenkovic et al., 2003).

Advanced glycation end-products (AGEs), derived from the reaction of reactive carbonyl- or dicarbonyl compounds with lysine or arginine groups on proteins (Münch et al., 1999), are present in β-amyloid plaques and NFTs (Wong et al., 2001; Reddy et al., 2002).

The first description of AGE in AD dates back to 1994, when it was demonstrated that plaque fractions of brains of AD patients contain higher levels of AGEs than age-matched control brains (Vitek et al., 1994). In a further study, the presence of AGE in NFTs and senile plaques has been elegantly demonstrated by immunohistochemical methods using antibodies specific for the defined AGEs pyrraline and pentosidine (Smith et al., 1994).

Although some authors have suggested that AGEs are very late markers (tombstones) of the disease (Mattson et al., 1995), it now widely accepted that they are active participants in the progression of the disease by rendering modified protein deposits insoluble and undegradeable, and by activating or enhancing the inflammatory response (Smith et al., 1995). In a further histochemical study, most senile plaques (including diffuse plaques) and cerebral amyloid angiopathy (CAA) from Alzheimer’s brains have been shown to exhibit AGE and ApoE immunoreactivity together. Interestingly, ~5% of plaques were AGE-positive but β-amyloid (Aβ)-negative. When the authors looked at NFTs, they found that AGEs were mainly present in intracellular NFTs, whereas ApoE was mainly present in extracellular NFTs (Sasaki et al., 1998). Moreover, when AGEs immunoreactivity was compared in tau of normal brains, preparations of soluble PHF-tau as well as insoluble PHF from AD brains, all three principal tau components showed AGE immunoreactivity in Western blots indicating that tau is glycated in PHF-tau. Moreover, immunoelectron microscopic analyses indicate that a CML antibody labels predominantly PHF in aggregates. Taken together, these results suggest that tau becomes AGE-modified in PHF-tau and that this is contributing...
to tangle formation in AD (Ko et al., 1999). In a further study, granules positive for Aβ, AGE and RAGE (one of the AGE receptors) were identified in the perikaryon of hippocampal neurons (especially from CA3 and CA4) in healthy and AD subjects. Interestingly, Aβ- and RAGE-positive granules were co-localized in one part of a single astrocyte, which led the authors to the conclusion that glycated Aβ is taken up via RAGE and is degraded through the lysosomal pathway in astrocytes (Sasaki et al., 2001).

It has been suggested that formation of AGE cross-links in these AD-specific lesions is an early step in seed formation similar to that found for Lewy bodies (Münch et al., 2000). AGE cross-linking also contributes to the insolubility and protease-resistance of such deposits (Bulteau et al., 2001; Sebekova et al., 1998).

Cell culture experiments have demonstrated that AGES exert direct toxicity to cells through predominantly apoptotic mechanisms (Artwohl et al., 2003; Kaji et al., 2003; Takeuchi et al., 2003). Furthermore, it was shown in retina neurons that apoptosis induced by the AGE precursor glyoxal was mediated by activation of caspase-3 (Reber et al., 2003). Some but not all studies suggest that neuronal death in AD is the result of an apoptotic mechanism (Jellinger and Stadelmann, 2001; Roth, 2001). However, the stereotypical manifestations that define the terminal phases of apoptosis, such as chromatin condensation, apoptotic bodies, and blebbing, are rarely seen in AD, but activated caspases, including caspase-3, are sometimes found in association degenerating neurons in AD (Gastard et al., 2003). In a further study, activated caspase-3 immunoreactivity was seen in neurons, astrocytes and blood vessels, was elevated in AD, and exhibited a high degree of colocalization with neurofibrillary tangles and senile plaques, suggesting that activated caspase-3 may be a factor in functional decline (Su et al., 2001). Therefore, the next step would be to confirm the colocalization AGES with apoptotic markers such as caspase-3 to determine if AGE deposition and caspase activation are causally linked.

nNOS is another marker that is uniquely expressed in the vulnerable pyramidal neurons in AD but not in healthy non-demented controls. nNOS was detected in neurons containing NFTs and those not yet affected by neurofibrillary degeneration. The involvement of nitric oxide (NO) in the pathological process of AD and the role of NO in oxidative stress has been suggested in earlier investigation of our group (Lüth et al., 2001, 2002).

In this study, AGE deposition was analyzed in the brains of young and old non-demented patients. The latter were compared with age-matched AD patients in early and late stages of the disease. In particular, AGES were co-localized with hyperphosphorylated tau in different stages of tangle formation and with markers of neurodegeneration and/or cell death (nNOS and caspase-3).

Materials and Methods

Cases

Brains used in the present study were obtained from 12 non-demented controls, subdivided into an older (65-90 years, five males, three females) and a younger (30-50 years, four males) group, as well as from 20 patients with AD, subdivided into an advanced (Braak stage V-VI; six males, four females) and early (Braak stage I-II; three males, seven females) stage of the disease (according to Braak and Braak, 1991). The presence of a dementia syndrome was defined in accordance with ICD-10 research criteria. Because of insufficient operationalization within ICD-10, the NINDS-AIREN criteria were additionally applied in all cases with a dementia syndrome (McKhann et al., 1984). The AD cases were matched with respect to age (mean age: aged controls, 79.3 ± 8.1 years; AD, 83.8 ± 6.6 years; P > 0.20, Student’s t-test), post-mortem interval (old controls, 64 ± 30 h; AD, 47 ± 33 h; P > 0.20) and the ‘Premortem Severity Index’ (PMSI) by Monfort et al. (1985) (P > 0.20) to minimize the likelihood of an artificial influence by premortem hypoxia and hypovolemia. The young control group (mean age = 25.6 years) was used to evaluate age-related changes as well as to compare with changes found in early-stage AD. The whole procedure of case recruitment, acquisition of patients’ personal data, performing the autopsy, and handling the autopic material was performed in accordance with the Helsinki Declaration in its latest version, and the convention of the council of Europe on Human Rights and Biomedicine. It was also approved by the Ethical Committee responsible for Leipzig University.

Brains of non-demented controls were obtained at routine autopsy from patients who died without a history of neuropsychiatric disorder, mental impairment or diabetes mellitus. There had to be clear evidence that the patient was alert, well oriented and capable of functioning relatively independently shortly before death. No pathological signs were detected by neuropathological examination. The clinical diagnosis of AD was based on the occurrence of significant intellectual dysfunction, i.e. the presence of deficits in at least four aspects of cognitive and social behaviour. Other causes of dementia were excluded by medical, psychiatric and paraclinical examination (following the Diagnostic and Statistical Manual of Mental Disorders, 3rd revised edn, DSM-III-R, American Psychiatric Association). Cases with a history of diabetes mellitus were excluded. Each case met the National Institute of Neurologic and Communicative Disorders and Stroke (NINCDS) and also the Alzheimer’s Disease and Related Disorders Association (ADRDA) criteria for definite diagnosis of Alzheimer’s disease, based on the presence of NFTs and neuritic plaques observed in the hippocampal formation and neocortical areas, as recommended (Braak and Braak, 1991).

Preparation of Tissue

Tissue blocks were taken from the auditory association area of superior temporal gyrus (Brodmann area 22), and immersed in 4% paraformaldehyde/0.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 4 days at 4°C. Blocks were subsequently immersed in 15% sucrose in phosphate buffered saline (PBS) for 24 h, followed by 30% sucrose in PBS for 48 h. Coronal sections, 30 µm thick, were cut on a freezing microtome and processed for the immunohistochemical detection of AGE, AT8, nNOS and caspase-3. The Gallyas staining method for demonstration of neuritic plaques, NFTs and neuritop threads was performed following the original description (Gallyas, 1971). Furthermore, the thioflavin S method was used for staging of the AD brains as described previously (Braak and Braak, 1991).

Immunohistochemistry

For immunohistochemistry, we have used the polyclonal AGE-antibody K 1936, as described previously (Wong et al., 2001), and the monoclonal AGE-antibody 4G9 which was directed against the defined AGE carboxymethyllysine (CML) (Castellani et al., 2001). In general, all AGE antibodies stain the same structures, including the somata of neurons and astroglia, blood vessels and neurite amyloid plaques. Since the antibody K 1936 stains AGES in astrocytes more intensely than the other AGE antibody, this antibody was used for most of the experiments. For double immunofluorescence experiments, the monoclonal AGE (CML) antibody 4G9 was also used. In detail, free-floating sections were briefly boiled in sodium citrate buffer (NaCl 150 mM, Na-citrate 100 mM, pH 6.0) and pre-incubated with 1% H2O2 for 30 min, followed by blocking of nonspecific binding sites with 5% normal goat serum (Sigma), 0.5% Triton X-100 (FERAK, Berlin, Germany) in 0.1 M Tris-buffered saline (TBS, pH 7.4) for 1 h. Next, sections were incubated with one of the following primary antibodies overnight at 4°C: polyclonal AGE-antibodies (K 1936 and K 2188) and one monoclonal CML antibody (4G9), polyclonal anti-nNOS (rabbit, anti-nNOS, Transduction Laboratories, 1:1000), anti-polycional AT8 (1:1000 diluted polyclonal anti-AT8, Innogenetics, Ghent, Belgium), anti-caspase-3 (1:1000 diluted polyclonal anti-caspase-3, Promega, Madison, WI) or anti-GFAP (1:1000).
diluted anti-human GFAP, clone 6D2, Dako). Primary antibodies were detected by (i) biotinylated goat anti-rabbit IgG or (ii) biotinylated goat anti-mouse immunoglobulin (1:1000, Dianova, Hamburg, Germany). Immunoreactivity was visualized with 3,3'-diaminobenzidine/H2O2. Primary antibodies were omitted in control incubations. For simultaneous detection of AGE and AT8, nNOS or caspase-3, sections were incubated with a cocktail containing the primary antibodies: monoclonal anti-AGE (monoclonal CML, antibody 4G9) and the corresponding polyclonal antibody (AT8, nNOS or caspase3). Immunodetection was carried out accordingly with a cocktail of the CyDye-labeled species-specific secondary antibodies goat anti-rabbit IgG-Cy2 (diluted 1:100, Dianova, Hamburg, Germany) and goat anti-mouse IgG-Cy3 (diluted 1:1000, Dianova, Hamburg, Germany). All incubation steps were separated by intensive washing with TBS. Sections were mounted and covered with Entellan (Merck, Darmstadt, Germany).

Quantitative Determination of AGE-positive Neurons and Astroglia

For quantitative determination of AGE-positive nerve cells in the young control group (n = 3, mean age = 25.6), the old control group (n = 3, mean age = 78.6), the early AD (n = 3, mean age = 81.6) and the late AD group (n = 3, mean age = 82.3), sections of a defined area of the cerebral cortex (Brodmann area 22) were immunostained for AGES. The percentage of AGE-positive neurons was determined by division of the number of AGE-positive cells by the total number of neurons (Nissl staining). AGE-positive astrocytes were quantified in a similar manner comparing them to the total number of GFAP positive astrocytes. Twelve sections were analyzed for each brain, and a computer-assisted microscopy system was used (Kontron Videoplan, Zeiss, Jena, Germany).

Results

Intracellular AGE Deposits in Nerve Cells Increase with Aging

In the cerebral cortex of healthy humans, a significant difference in the number of AGE deposits between the different age groups could be detected (Figs 1A,B and 2A,B). AGE deposition was detected in some subpial astroglia, and in a few blood vessels and nerve cells (0.4%, see Table 1) in the cerebral cortex of the young (25.6 years) control group (Table 1, Figs 1A and 2A). The number of AGE-positive nerve cells was increased to...
24.6% in the group of older (78.4 years) non-demented humans (Table 1, Figs 1B and 2B). Furthermore, AGE deposits in blood vessels and in astrocytes could be observed in increasing numbers in the ‘old’ group (Fig. 1B, arrow head). This increase in AGE immunostaining was visible in both the number of AGE immunoreactive neurons and in the intensity of the local deposits (Table 1). AGEs were very rarely observed in astrocytes of the subpial region in brains of young humans (Fig. 1A). The intensity of the immunoreaction in astrocytes in the subpial region increased continuously with age, and was highest in old human brains (Fig. 1B).

### Intracellular AGE Deposits Increase in Brains of Alzheimer’s Patients Compared with Age-matched, Non-demented Controls

The deposition of AGEs in brains of patients with both early (I–III) and late (V and VI) stages of AD were compared with age-matched, non-demented brains with respect to the number of AGE-positive neurons and their intensity. The numbers of AGE-positive neurons were clearly increased in AD brains (from 37.5% in early AD and 72.6% in late AD), compared with non-demented brains of the same age (24.6%; Table 1). The even more obvious difference between AD patients and controls was the occurrence of senile plaques in great quantities in AD brains, which were accompanied by AGE-positive astrocytes (Figs 2A–D and 3). Besides astrocytes in the subpial regions, many astrocytes around senile plaques showed intense AGE immunoreactivity (Fig. 3C,D). Notably, AGEs could be detected

| Table 1 | Semiquantitative determination of the percentage of AGE-positive neurons compared with the total number of neurons (visualized by Nissl stain) in the cerebral cortex (area 22) of controls of different ages, and of cases with early and late stages of AD |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Young control group | Old non-demented control group | AD (early stage) | AD (late stage) |
| Mean age (years) | 25.6            | 78.6            | 81.6            | 82.3            |
| Nissl stained cells (no. of cells/mm²) | 1934 ± 180 | 1791 ± 166 | 1683 ± 179 | 1574 ± 230 |
| AGE-positive cells (no. of cells/mm²) | 8.5 ± 3.4 | 441 ± 46 | 631 ± 42 | 1142 ± 7.2 |
| AGE-positive cells as % of Nissl-stained cells | 0.43 ± 0.17 | 24.6 ± 2.6 | 37.5 ± 2.5 | 72.6 ± 4.6 |

Data were collected from 10 sections of three patients for each group.
in astrocytes that were in physical contact to amyloid plaques, and also in those farther from the plaques, indicating that soluble factors may contribute to intracellular AGE formation in astrocytes. Furthermore, AGE immunostaining could also be observed in blood vessels and glial cells in the white matter of the cerebral cortex of AD patients, but not in non-demented controls (Fig. 4).

**Figure 3.** AGE-positive astrocytes in the layer I and II and in deeper layers of the cerebral cortex of control and AD brains. AGE-positive astrocytes were detected in the cerebral cortex (layers I and II) of a non-demented old control (A) and an AD brain (B). Astrocytes in layers V/VI of a non-demented control (C) and AD brain (D) were also immunopositive for AGES. In AD brains, AGE-positive astrocytes were located around senile plaques (D), while they were more isolated in control brains (C). Scale bar = 20 μm.

**Figure 4.** Demonstration of AGE-positive glial cells in the white matter of the cortex of a control and an AD case. In the white matter of the ‘old’ control brains, AGE immunoreactivity was only observed in blood vessels (A). In contrast, AGES were also detected in astrocytes of the white matter in the AD brains (B). Scale bar = 20 μm.

**Deposition of AGES in Brains of AD Patients Increased with Braak Stage**

The progression of AD pathology can be analyzed and described by the six different AD stages introduced by Braak and Braak (1991). Brains were staged by analysis of fibrillar amyloid plaques (thioflavin S method) and by analysis of NFTs (immuno- reaction of the antibody AT8). Using this classification system,
the stage-dependent deposition of AGEs was determined in more detail in AD patients with defined Braak stages. Unexpectedly, deposition of AGEs in astrocytes, neurons and blood vessels could be observed in this area in Braak stages I and II (Fig. 1 C), in which the neurofibrillary pathology is still limited to the entorhinal cortex, and has not spread to the investigated Brodmann area 22 (which is a part of the unaffected isocortex). The presence of markers of ‘carbonyl stress’ (increased levels of AGEs) in areas yet unaffected by AD-specific lesions is reminiscent of data obtained by Smith and Perry, where markers of ‘oxidative stress’ were shown to precede tangle and plaque formation in AD (Perry et al., 2002). The deposits quite clearly increased in intensity over stages IV–VI (Figs 1 D and 2 C, D). In the late stages, the number of AGE-positive neurons was extremely high (~72% of neurons) (Figs 1 D and 2 D, Table 1). AGE-positive astroglia, associated with the increased number of senile plaques, also increased in number and intensity of their immunoreactivity (Figs 1 C, D and 2 C, D).

**Co-localization of AGES with Different Hallmarks of AD AGES and Hyperphosphorylated Tau (AT8 Immunoreactivity)**

As outlined in the introduction, NFTs go through three different stages of development (stage 1, pre-NFTs; stage 2, intraneuronal NFTs; and stage 3, extra-neuronal NFTs; Braak et al., 1994; Augustinack et al., 2002). All three types of tangles can be present in the same region of an AD patient simultaneously. In the brains used in this study, hyperphosphorylated tau (AT8 immunoreactivity) was detected in neurons in various stages of tangle development, including those exhibiting a diffuse AT8 immunoreactivity in the perikaryon (and representing neurons in the pre-neurofibrillary tangle stage). Using double immunofluorescence to co-localize AGES and hyperphosphorylated tau, AGE immunoreactivity could be detected in neurons in tangle stage 1, i.e. in neurons with pre-NFTs (Fig. 5). In these neurons, AT8 immunoreactivity was nonfibrillar and diffusely distributed in the cytoplasm (Fig. 5). It has to be noted that these diffusely AT-8 positive neurons were always AGE-positive, indicating that hyperphosphorylation and AGE accumulation may be linked. Also, in tangle stage 2, the majority (~70%) of nerve cells contained AGES and AT8 immunoreactivity, whereas extraneuronal NFTs (tangle stage 3) very rarely showed AGE immunoreactivity (Fig. 5 A–C).

**AGE and nNOS**

Various isoforms of nitric oxide synthase are elevated in AD, indicating a critical role for NO in the pathomechanism. In the isocortex of normal human brains, nNOS immunoreactivity has been found in two different types of interneurons, whereas isocortical pyramidal cells were generally free of immunoreactivity (Lüth et al., 2000). Those nNOS-positive interneurons were identified in previous studies as Martinotti neurons in the rat and human cerebral cortex (Hedlich et al., 1990; Lüth et al., 1994). While the distribution and immunoreactivity of cortical interneurons expressing nNOS was unchanged in AD, nNOS was detectable in pyramidal neurons of the isocortex (Fig. 6 A). Both tangle-bearing neurons and pyramidal neurons apparently unaffected by neurofibrillary degeneration were clearly nNOS positive. Aberrant expression of nNOS in cortical pyramidal cells was highly co-localized with nitrotyrosine, indicating that the protein was not only deposited but enzymatically active (Lüth et al., 2002). In this study, double immunofluorescence of AGE and nNOS showed a co-localization of AGES and nNOS in many but not all pyramidal cells in upper layers II and III and deep layers V and VI (Fig. 6 A, B). nNOS was observed in cortical interneurons; however, no AGE depositions were observed there (Fig. 6 C–E).

**AGE and Caspase-3**

Activated caspase-3 has the properties of a cell death executioner protease and has been seen in neurons, astrocytes, and in blood vessels in AD. It also exhibits a high degree of co-localization with NFTs and senile plaques. This suggests that activated caspase-3 may be a factor in functional decline and may have an important role in neuronal cell death in an AD brain. As described by other groups, the percentage of apoptotic neurons in the investigated Brodmann area 22 was quite small (<1%). Among these few caspase-3 positive neurons, a co-localization of AGE with caspase-3 immunoreactivity could be observed in ~75% of the neurons (Figs 7 A–C). However, the majority of neurons were positive for either AGE or caspase-3 alone. Similar results were obtained by the combination of AT8 and caspase-3 immunoreaction. Some neurons were double labeled with AT8 and caspase-3 (Fig. 7 D–F), whereas other neurons show AT8 or caspase-3 immunoreactivity alone (Fig. 7 D–F).

**Discussion**

The aim of this study was to examine the normal age-dependent deposition of AGEs in the human brain, the comparison of these data with those in AD, and a potential co-localization with markers of such as nitrooxidative stress and neurodegeneration such as nNOS and caspase-3. The study was expected to deliver more information about the difference of AGE-deposition between normal aging and AD, and about a possible pathogenic role of AGES in formation of AD specific lesions.

In healthy, non-demented humans, an age-dependent increase could be detected in both the percentage of

---

**Figure 5.** Co-localization of AGES and hyperphosphorylated tau (AT-8). Double immunofluorescence was used for the simultaneous detection of AGES (A, green) and hyperphosphorylated tau (AT-8 antibody) (B, red). Double-labeled structures appear in yellow in (C). AGES and hyperphosphorylated tau immunoreactivity was detected in neurons with pre-NFTs (see arrow in A–C). Other tangle bearing neurons and the neuropil treads (arrow head) were only immunoreactive for hyperphosphorylated tau. Scale bar = 50 μm.

**Figure 6.** Co-localization of AGES and nNOS. Double immunofluorescence was used for the simultaneous detection of nNOS (A, C, red) and AGES (B, D, green). Most pyramidal neurons of the cerebral cortex which show nNOS immunoreactivity were also AGE-positive. nNOS in the human cerebral cortex of patients with AD was localized in two types of neurons: pyramidal cells (C, arrow head) and interneurons (C, asterisk). AGES were co-localized with nNOS only in the pyramidal cells, but not in interneurons (E). Furthermore, not all AGE-immunopositive pyramidal cells show co-localization with nNOS (arrow in D and E). Scale bar = 50 μm.

**Figure 7.** Co-localization of caspase-3 with hyperphosphorylated tau (AT8) or AGES. Double immunofluorescence pictures of caspase-3 (A) with AGE (B) and caspase-3 (D) with hyperphosphorylated tau (E) in the cerebral cortex of an AD patient is shown. Caspase-3 (A, green) was co-localized with AGES (B, red) in ~75% of the caspase-3 positive neurons (arrows in C). Some neurons were also labeled with hyperphosphorylated tau and caspase-3 (D–F, arrows in F). Scale bar: 50 μm.
Advanced Glycation End Products in Aging and Alzheimer’s Disease  

AGE-positive neurons and the intensity of the deposits. Our data support previous studies where a positive correlation between AGE-immunoreactivity in hippocampal pyramidal neurons and age was demonstrated. In this study, a more intense immunoreaction was observed in the more vulnerable CA3-4 pyramidal neurons compared with that of the more resistant CA1 neurons (Kimura et al., 1996). It is not clear which proteins or larger structures of the cell were actually modified by AGEs. However, neuronal AGEs that form granular deposits were associated with lipofuscin (Takeda et al., 2001). Lipofuscin is constituted not only of lipid peroxidation products but of AGEs which may be the origin of fluorescent pigments (Horie et al., 1997). Humans are not the only species where an age-dependent accumulation of AGEs is observed. In dogs, distinct AGE-positive granules can be detected in Purkinje cells as occurs in humans, and accumulate in an age-dependent manner (Weber et al., 1998).

A dramatic increase in the number of AGE-loaded astrocytes could also be observed in the aging brain, whereas AGES were very rarely observed in astrocytes of the subpial region in brains of young humans. The number that were immunoreactive in astrocytes in the subpial region increased continuously with age, and were highest in old human brains (Fig. 1B). It was interesting to observe that these AGE-positive astrocytes first occurred at the brain–cerebrospinal fluid (CSF) border, which may suggest that AGES or their precursors were taken up or diffused from the CSF into the subpial region and were then taken up by these astrocytes. Alternatively, factors from the CSF may activate astrocytes to produce AGES via activation of the myeloperoxidase system (Anderson et al., 1999).

A more general reason for the observed increase in AGE levels in both cell types could be caused by higher levels of their precursors, such as glyoxal and methylglyoxal. Methylglyoxal levels could be caused to increase by the higher levels of triosephosphates which accumulate under impaired glycolytic flow through the TCA cycle and the mitochondrial chain (Beiswenger et al., 2003), a condition called ‘pseudohypoxia’ in diabetes (Ido and Williamson, 1997). Another possible reason could be the lower capacity of the glyoxalase system for the detoxification of these compounds, e.g. by a depletion of its rate-limiting cofactor glutathione (Vander Jagt and Hunsaker, 2003).

We have shown previously in humans and in transgenic APP-overexpressing mice that AGES were detected in astrocytes that were in physical contact with amyloid plaques (Wong et al., 2001; Münch et al., 2003). However, we demonstrate in this study that astrocytes further away from plaques also accumulate AGES, indicating that soluble factors may also contribute to AGE formation. AGE immunostaining could be observed in and has been consistently reported in blood vessels, in particular, in vessels of diabetics and smokers (Lyons, 1993; Nicholl et al., 1998).

There is no obvious explanation why AGE deposition increases in AD. It could be a consequence of the disease, or could be a part of its cause. The latter hypothesis is supported by the fact that in Braak stages I and II, where neurofibrillary pathology is limited to the entorhinal cortex, the deposition of AGES in astrocytes, nerve cells and blood vessels could be observed in the isocortex (Brodmann area 22). Foremost, and importantly, these data suggest that carbonyl stress precedes hyperphosphorylation and tangle formation. One possible link between carbonyl stress and hyperphosphorylation (as a prerequisite of tangle formation) could be the activation of tau kinases by reactive carbonyl compounds (AGE precursors). For example, methylglyoxal and acrolein activate p38 MAP kinase in a dose-dependent manner, increasing the phosphorylation of tau at the site recognized by PHF-1 (Reynolds et al., 2000; Liu et al., 2003).

In support of this hypothesis, tau hyperphosphorylation by the stress-inducible p38 kinase has also been reported to be present in neurons in AD (Zhu et al., 2000). Furthermore, reactive carbonyl compounds are known to inhibit phosphatases (Portero-Otin et al., 2002). The discussion about AGES as being early or late markers of AD is very much reminiscent of the longstanding discussion about oxidative stress as being an early or late event in the disease. As with AGES, more recent results indicate that increased oxidative damage really is an early event in AD that even decreases with disease progression and lesion formation (Smith et al., 1997; Nunomura et al., 2001; Pratico et al., 2002). Since some of the precursors (e.g. glyoxal) or end-products (carboxymethyl-lysine) of glycation and (radical-induced) lipid peroxidation reactions are identical, it is quite obvious that both markers are early markers of the disease.

We have shown previously that the presence of nNOS and nitrotyrosine in pyramidal neurons of the isocortex in a specific marker for AD (Lütth et al., 2001, 2002). In our study, AGE and nNOS are often co-localized which might suggest that they could be causatively linked. Activated caspase-3, a cell death executioner protease, exhibits a high degree of colocalization with NFTs and senile plaques in AD.

Although the total number of caspase-3 positive neurons was <1%, we have observed a co-localization of caspase-3 immuno-reactivity with AGE in ~75% of the neurons. However but the majority of neurons are positive either for AGE or for caspase-3 only. Since the percentage of apoptotic neurons in area 22 in the AD patients was <1%, it is not possible to prove a causal link between AGE deposition and apoptosis via the caspase-3 pathway. However, other groups have suggested such a biochemical link between AGE accumulation, nNOS expression and apoptosis. In this study, Cellek and Moncada showed that AGES, but not high glucose concentrations, led to caspase-3-dependent apoptosis in SH-SY5Y neuroblastoma cells but only in those differentiated neurons expressing nNOS (Cellek et al., 2003).

Notes
This study was supported by the Bundesministerium für Bildung, Forschung und Technologie (BMBF), Interdisciplinary Centre for Clinical Research at the University of Leipzig (01KS9504, Project C1 and N1), the Alzheimer Forschungs Initiative e.V. (AFI), the BrainNet (Deutsches

Table 2
Semiquantitative determination of the percentage of AGE-positive astrocytes compared with the total number of GFAP-positive astrocytes in the cerebral cortex (area 22) of controls of different ages, and of cases with early and late stage of AD

<table>
<thead>
<tr>
<th>Group</th>
<th>Young control group</th>
<th>Old non-demented control group</th>
<th>AD (early stage)</th>
<th>AD (late stage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>25.6 ± 7.6</td>
<td>78.6 ± 10.3</td>
<td>81.6 ± 5.1</td>
<td>82.3 ± 4.3</td>
</tr>
<tr>
<td>GFAP-positive astrocytes (nc. of cells/mm²)</td>
<td>71 ± 26</td>
<td>101 ± 34</td>
<td>127 ± 51</td>
<td>173 ± 43</td>
</tr>
<tr>
<td>AGE-positive astrocytes (nc. of cells/mm²)</td>
<td>18.6 ± 9.2</td>
<td>36.1 ± 10.3</td>
<td>82.7 ± 20.5</td>
<td>108 ± 15.8</td>
</tr>
<tr>
<td>AGE-positive astrocytes as % of GFAP-positive astrocytes</td>
<td>26.1 ± 12.9</td>
<td>35.7 ± 10.2</td>
<td>65.1 ± 16.1</td>
<td>62.4 ± 9.1</td>
</tr>
</tbody>
</table>

Data were collected from 10 sections of three patients for each group.


