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Immunochemical crossreactivity of antibodies specific for "advanced glycation endproducts" with "advanced lipoxidation endproducts"

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Abstract

Antibodies against advanced glycation endproducts (AGEs) are used for their immunohistological localization in tissues, for example in Alzheimer's disease (AD) or diabetes. Many monoclonal and polyclonal antibodies have been used, and their specificity is unknown in most cases. Increased radical production, leading to the formation of lipid-derived reactive carbonyl species, such as malondialdehyde (MDA), acrolein, and glyoxal, is a characteristic aspect of age-related diseases like Alzheimer's disease or diabetic polyneuropathy. These reactive carbonyl species are able to modify proteins, resulting in AGE related structures, termed "advanced lipoxidation products" (ALEs). In this study, the monoclonal carboxymethyllysine-specific antibody 4G9 and the polyclonal AGE-antibody K2189 were tested for their immunoreactivity towards these carbonyl-derived protein modifications. To investigate which carbonyl-modified amino acid side chains are specifically recognized by these antibodies, peptide membranes were incubated with glyoxal, MDA and acrolein. As model proteins, microtubuli associated protein tau (MAP-tau), β-amyloid, human serum albumin and chicken egg albumin were incubated likewise. It was found that both antibodies detected reaction products of these carbonyl compounds on lysine- and arginine residues and for the protein modification, it was found that some epitopes might not be detected. In conclusion, AGE-antibodies might not only detect sugar-derived AGEs but also structures derived from lipid peroxidation products (serving as markers of oxidative stress).

Keywords: Advanced glycation endproducts; Advanced lipid peroxidation endproducts; Carbonyl stress; Glycation; Alzheimer's disease; Oxidative stress

1. Introduction

The reaction of reducing sugars or sugar fragmentation products with free nucleophilic protein side chains, such as those of arginine and lysine, belongs to the most common non-enzymatic posttranslational modifications of proteins (Fig. 1A). Subsequent rearrangements, oxidations and dehydrations yield a heterogeneous group of mostly colored and fluorescent compounds termed 'Maillard products' or advanced glycation endproducts (AGEs) (Fig. 1B–D). Most notably, AGEs have been observed on long-lived proteins such as collagen, eye lens crystalline and also in pathological protein deposits such as β-amyloid plaques or neurofibrillary tangles in Alzheimer's disease (AD) and β_2-microglobulin deposits in hemodialysis patients [18]. Since they also upregulate a variety of growth factors and cytokines by a receptor-mediated mechanism, AGE-modified proteins are proposed to be involved in the pathology and etiopathogenesis of various age-related diseases including Alzheimer's disease and complications of diabetes and hemodialysis [22,33,30]. Thus, this state of carbonyl overload with potential damage to proteins and DNA is termed "carbonyl stress".

Excess production of oxygen free radicals ("oxidative stress") is another hallmark of age-related and neurodegenerative diseases like Huntington's Chorea [3], Parkinson's disease [13], Alzheimer's disease [4,23] or amyotrophic lateral sclerosis [27]. Reactive oxygen species (ROS) includ-

Abbreviations: AD, Alzheimer's disease; AGE, advanced glycation endproduct; ALE, advanced lipoxidation endproduct; BSA, bovine serum albumin; CEL, carboxy-ethyl lysine; CML, carboxy-methyl lysine; DOLD, desoxyglucosone lysine dimer; FDP-lysine, *N*-(3-formyl-3,4-dehydropiperidino)lysine; FFI, furyl furfuryl imidazolone; GOLD, glyoxal lysine dimer; KLH, keyhole limpet hemocyanin; MAP-tau, microtubuli associated protein tau; MDA, malondialdehyde; MOLD, methylglyoxal lysine dimer; ROS, reactive oxygen species; TBS, Tris buffered saline

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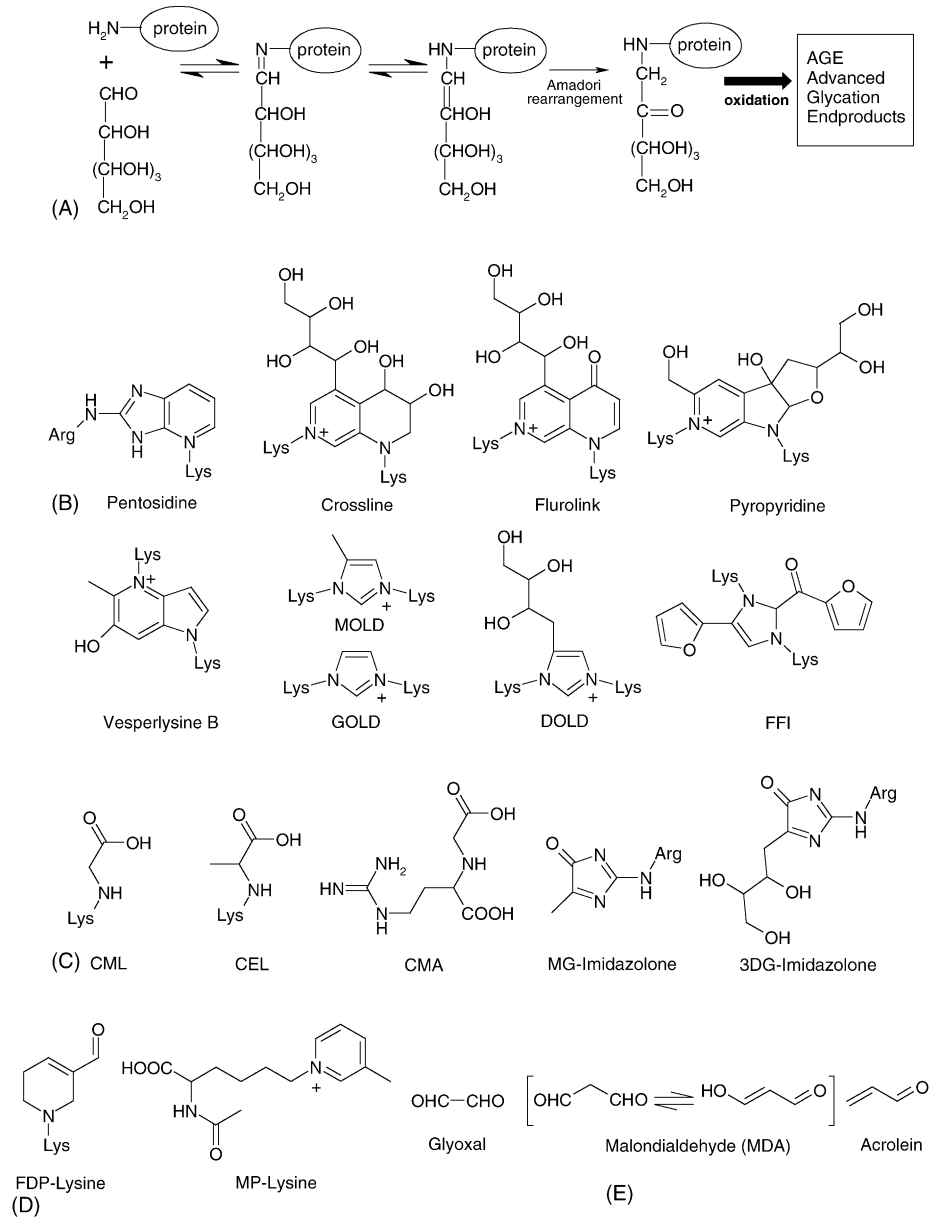

Fig. 1. Chemical structures of AGEs, ALEs and routes to their formation. Formation of AGEs: After the Amadori rearrangement (A), the reaction is irreversible and leads (after subsequent oxidation steps) to fluorescent, often crosslinked AGEs (B), to non-fluorescent AGEs (C), or to FDP-lysine and MP-lysine (derived from the reaction of acrolein with lysine) (D). The reactive carbonyl compounds glyoxal, MDA and acrolein, which can all be derived from lipid peroxidation, are also shown (E).

ing superoxide react with unsaturated lipids in the lipid bilayer of cells and generate a number of secondary products, both from fission and endocyclization of oxygenated fatty acids that possess neurotoxic activity [19]. This process is called “lipid peroxidation” and leads to the formation of reactive dicarbonyl compounds (such as glyoxal and malondialdehyde (MDA)) and α,β-unsaturated aldehydes (such as acrolein or hydroxynonenal) [15,31] (Fig. 1E). Like glucose, a well-known precursor for AGE-formation, these compounds are aldehydes. Glyoxal is particularly reactive due to the presence of an adjacent carbonyl group, acrolein due to a double bond conjugated to the C=O bond. It is thus obvious, that modification of proteins by glucose is relatively slow compared with modification by small dicarbonyl compounds such as derived from lipid peroxidation.

Although research into AGEs has been going on for decades, only a small fraction of the many different AGE structures have been identified. AGEs can be divided into heterocyclic compounds (structurally related to pentosidines and imidazolones) and short-chained non-cyclic entities (Fig. 1B and C). Many monoclonal and polyclonal antibodies have been raised against AGE-modified proteins, most of them against proteins incubated with high concentrations of glucose for extended periods. Since the antigenic

structures of AGEs are heterogeneous, the specificity of AGE-antibodies is undefined in many cases, and thus immunohistochemical localization of AGEs in tissues or their quantitative determination in biological fluids sometimes yields inconsistent results among different investigators. Thus, a systematic mapping of their epitope recognition pattern would help in the evaluation of these different results. In a first attempt, AGE antibodies have been classified as group I as specific for carboxymethyl lysine (CML) and groups II and III as specific for other non-CML epitopes [11]. Using a combinatorial dipeptide library, we have characterized six different AGE antibodies with respect to their immunoreactivity towards glucose-derived AGE-modified dipeptides. We have identified arginine- and lysine-derived AGEs, but also some epitopes on asparagine and on heterocyclic amino acids as immunoreactive towards different AGE-antibodies [7].

Since advanced lipoxidation end products (ALEs), derived from the reaction of lipid peroxidation products with proteins [1,17], could share similar structures with AGEs, we speculated if they could be detected by "conventional" AGE-antibodies. To obtain first answers to the question, four proteins and a dipeptide library were modified by MDA, glyoxal or acrolein, and the recognition pattern of these ALEs by two different AGE-antibodies was investigated.

2. Materials and methods

2.1. Materials

Since commercial chicken egg albumin is spray-dried and AGE-modified to some extent, it was necessary to prepare fresh protein from eggs. Briefly, egg white was separated and solved in saturated (767 g/l) ammonium sulfate solution. The supernatant was decanted after centrifugation and 1 N sulfuric acid was added to adjust pH 4.6. The precipitated albumin was solved in aqua dest., and dialyzed against aqua dest. for 12 h. After dialysis, the albumin solution was sterile filtrated through 0.22 μm filters. HSA was purchased from Sigma-Aldrich (Steinheim, Germany). Recombinant human microtubuli associated protein tau (MAP-tau) protein was provided by T. Grune (Charité, Berlin, Germany). β-Amyloid peptide (1–40) was synthesized as described previously [16].

2.2. Methods

2.2.1. Immunohistochemistry of human brain tissue

For this investigation, selected slices from the temporal cortex of three human AD brains (two males, one female) from the collection of the Department of Neuroanatomy of the Paul Flechsig Institute of Brain Research, University of Leipzig (Germany) were used. Case recruitment, acquisition of patients' personal data, performing the autopsy, and handling the autoptic material has been approved by the responsible Ethical Committee of Leipzig University. 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 behavior. Other causes of dementia were excluded by medical, psychiatric and paraclinical examination (Diagnostic and Statistical Manual of Mental Disorders, DSM-IIIR, American Psychiatric Association). Each case met the National Institute of Neurologic and Communicative Disorders and Stroke (NINCDS) and 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 [18,36]. Tissue blocks were taken from the temporal cortex (Brodmann area 22) were 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. For immunohistochemistry, the polyclonal AGE-antibody K2189 and the monoclonal AGE-antibody 4G9, which is directed against the defined AGE CML, were 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% H_2O_2 for 30 min, followed by blocking of unspecific binding sites with 5% normal goat serum (Sigma-Aldrich, Taufkirchen, Germany), 0.5% Triton X 100 (FERAK, Berlin, Germany) in 0.1 N 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: (a) a polyclonal AGE-antibodies (K 2189, 1:500) or (b) a monoclonal CML antibody (4G9, 1: 500). Primary antibodies were detected by (a) biotinylated anti-rabbit Ig**G** in combination with Extravidin-peroxidase conjugate (1:1000, Sigma), or (b) biotinylated anti-rabbit Ig**G** in combination with Extravidin-peroxidase conjugate (1:1000, Sigma). Immunoreactivity was visualized with 3,3′-diaminobenzidine/H_2O_2.

2.2.2. Modification of proteins with carbonyl compounds

Recombinant MAP-tau, β-amyloid, human serum albumin and chicken egg albumin were incubated at a concentration of 1 mg/ml in PBS with 1 mM glyoxal, 1 mM MDA, or 1 mM acrolein for 72 h at 37 °C.

2.2.3. Determination of AGE-antibody immunoreactivity of carbonyl modified proteins by dot blot

One microliter solution containing 30 ng of MDA-, glyoxal- and acrolein-modified protein MAP-tau, β-amyloid, human serum albumin or chicken egg albumin as well as unmodified control protein were applied as a spot to a nitrocellulose membrane. The membranes were allowed to dry and blocked for 1 h with 0.5% bovine serum albumin

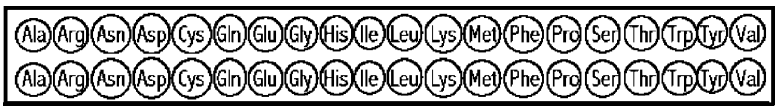

Fig. 2. Set-up of the peptide library containing 20 amino acids. The 20 spots of the peptide library contain one β-alanin-spacer (C-terminus) and one of the 20 amino acids (N-terminus). One peptide strip containing two rows of 20 peptides was used for each experiment. The triple amino acid code is used.

(BSA)/Roti-Block (Roth, Karlsruhe, Germany). Membranes were incubated with the antibodies K2189 or 4G9 at 4 °C at a dilution of 1:1000 in TBS overnight. Membranes without primary antibody served as negative controls. The membranes were washed 3× for 5 min in TBS, 15 min in TBS-Tween (0.1%), 3× for 5 min in TBS, incubated with swine-anti-rabbit (for K2189) and rabbit-anti-mouse (for 4G9) horseradish peroxidase conjugated IgG (Dako, Glostrup, Denmark) respectively, at a dilution of 1:1000 for 1 h at room temperature and washed again. Color was developed on the spots by using the Peroxidase Substate Kit DAB, SK-4100 (Vector Laboratories Inc, Burlingame, USA), according to the manufacturer instructions. After drying for 2 h, the immunoreactivity of each spot was determined by scanning of the dot blot, converting the picture to grayscale and analyzing the intensity of each of the dots by NIH Image 1.63. The relative intensity of each spot was calculated by dividing the intensity of the area within a spot by the intensity of the area of background.

2.3. Modification of peptide libraries with carbonyl compounds

Peptide libraries on filter paper sheets were synthesized and N-terminally acetylated as described previously [20]. Peptides were synthesized in an array of 10 × 20 spots (Fig. 2). Two rows of a peptide membrane were used for each experiment. They were incubated with PBS (as a negative control), or with 1 mM glyoxal in PBS, 1 mM MDA in PBS and 1 mM acrolein in PBS at 37 °C for 3 days. To remove excess carbonyl compounds, membranes were washed five times with PBS for 15 min.

2.3.1. Determination of antibody immunoreactivitiy on peptide libraries

The membranes were blocked for 1 h with Roti-Block (Roth, Karlsruhe, Germany). Then the membranes were incubated with the antibodies K2198 or 4G9 at 4 °C at a dilution of 1:1000 in TBS for 12 h. As a control, membranes were treated under the same conditions without primary antibodies. The membranes were washed 3× for 5 min in TBS, 15 min in TBS-Tween (0.1%) and 3× for 5 min in TBS and incubated with swine-anti-rabbit (for K2189 and control) and rabbit-anti-mouse (for 4G9 and control) horseradish peroxidase conjugated IgG (Dako, Glostrup, Denmark) at a dilution of 1:1000 at room temperature for 1 h. After washing with TBS and TBS-Tween as described above, they were subjected to immunoblotting without drying. The ECL Western blotting detection reagents RPN2106 (Amersham Pharmacia Biotech, Freiburg, Germany) was used for detection of immunoreactivity. Using the 'Fujifilm LAS-1000' luminescence detection camber, the intensity of the spots was quantified after subtraction of the background.

3. Results

3.1. Detection of AGE-positive structures in AD brain by two different AGE antibodies

4G9 is a monoclonal antibody, which was raised against keyhole limpet hemocyanin (KLH)-AGE (highly modified with glucose), and then screened on CML-recognition. K2189 is a polyclonal rabbit antibody, which was also raised against KLH-AGE. As previously described by our and other groups, AGEs can be detected in defined structures such as the cytoplasm of astroglia and neurons (sometimes as diffuse staining, sometimes as tangle-like structures) in the AD brain [5,12]. Furthermore, they can be identified in amyloid plaques and blood vessels [6,37]. In a first set of experiments, we investigated whether the antibodies K2189 and 4G9 stain these characteristic structures similar to previously used antisera. Our study shows, that both antibodies recognize AGE-positive structures in the cytoplasma of pyramidal neurons and astroglia (Fig. 3A, D, B and E). In addition, amyloid plaques and blood vessels walls are also positive for AGEs (Fig. 3C and F). No obvious difference in the overall staining pattern could be detected between the monoclonal 4G9 (Fig. 3A–C) and the polyclonal K2189 (Fig. 3D–F) AGE antibody.

3.2. Immunoreactivity of AGE-antibodies with ALE-modified proteins

Both AGE-antibodies were produced by using proteins maximally-modified with glucose as antigens. However, not much is known about the crossreactivity of such AGE-antibodies with other aldehyde-derived protein modifications. To investigate whether proteins modified by reactive carbonyls can also be detected with AGE antibodies, four proteins were immunostained after they had been modified by glyoxal, MDA or acrolein (Fig. 4). β-Amyloid (the major component of senile plaques), MAP-tau (the main protein in neurofibrillary tangles), HSA and ovalbumin (model proteins) were employed. Glyoxal-modified albumin, HSA, MAP-tau and β-amyloid were all strongly recog-

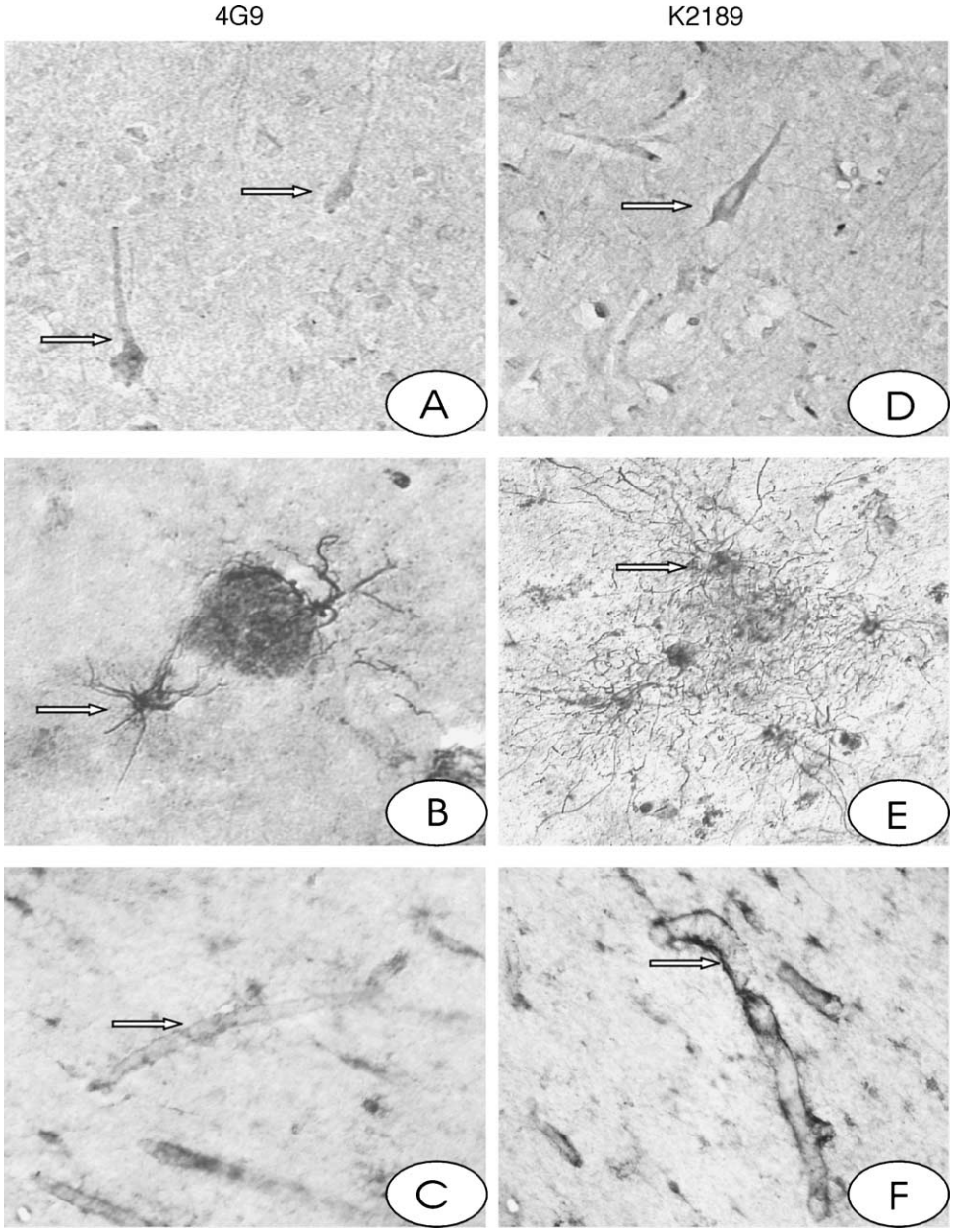

Fig. 3. Immunohistochemical staining of human AD brain with the AGE-antibodies 4G9 and K2189. Immunohistochemical staining of Brodmann area 22 of the cerebral cortex of an AD patient with the two AGE-antibodies. (A)–(C) and (D)–(F) shows the immunoreactivity with the antibodies 4G9 and K 2189, respectively. In general, both AGE antibodies demonstrate the same structures in the AD brain, e.g., nerve cells, predominantly pyramidal neurons (A and D), plaques and plaque associated astroglia (B and E), and blood vessels (C and F).

nized by both antibodies (4–5-fold of background intensity). A strong immunoreactivity against MDA-modified proteins could only be detected with MAP-tau, but not with the other proteins. 4G9 was able to recognize MDA-modified HSA, but with less intensity (2.2-fold of background intensity). Acrolein-modified proteins were also slightly immunostained, but this was only significant for MAP-tau. When the primary antibody was omitted, no signals could be detected, indicating that non-specific binding of the secondary antibodies can be excluded. However, our experiments indicate that protein modification of certain proteins with glyoxal, but also with MDA or acrolein might lead to positive recognition of such protein in histochemical experiments.

3.3. Immunoreactivity of antibodies with ALE-modified peptide libraries

To further characterize the antigenic epitopes of dicarbonyl-modified proteins, it was necessary to elucidate which amino acid side chains react with reactive carbonyls and is then recognized by AGE antibodies. Very interestingly, both antibodies predominantly recognized

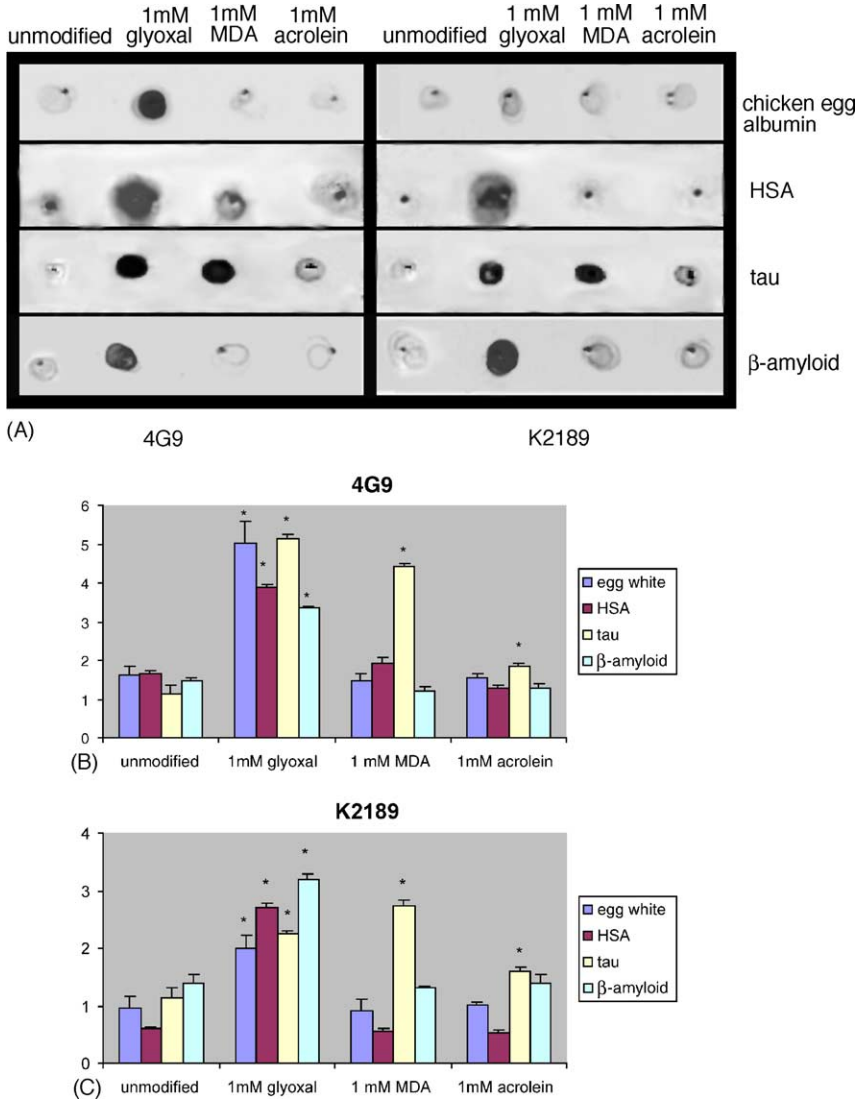

Fig. 4. Recognition of glyoxal, MDA or acrolein-modified proteins on dot blots. Unmodified chicken egg albumin, HSA, MAP-tau and β-amyloid and modifications of the proteins with glyoxal, MDA and acrolein in concentrations of 1 mM were spotted on nitrocellulose membranes and immunostained with the antibody 4G9 or the antibody K2189. Glyoxal modifications are always strongly immunoreactive, whereas the immunoreactivity of MDA- and acrolein-modifications depend on the protein and the antibody. A typical dot blot is shown in (A), its densitrometric analysis in (B) and (C). The experiments were done in triplicate (asterisk indicates a significant difference compared to the unmodified protein ($P < 0.05$)).

carbonyl-modified lysine and arginine residues (Figs. 5 and 6). Acrolein-modifications gave the most intensive signals, followed by glyoxal and MDA. For K2189, additional immunoreactivity can be detected for the aromatic amino acids tyrosine and phenylalanine and also for the hydrophobic amino acids valine, methionine, and isoleucine.

When the intensity of the immunostained spots between 4G9 and K2189 were compared quantitatively, they differed between the two antibodies because of the difference in background staining. Although the synthetic blocking reagent Roti-Block (Roth, Karlsruhe, Germany) was used for blocking of the membranes instead of BSA (which is AGE-modified to some degree), a significant background staining of the membranes was still observed. Because of the high non-specific background staining with the antibody 4G9, the relative intensities reached only 1.4, whereas a value of 14 can be observed with the antibody K2189. When incubations without the primary antibody or with unmodified membranes were carried out, no signals were detected, which indicates that the immunoreactivity is not caused by the secondary antibody or by a reaction of the primary antibody with the amino acid itself.

4. Discussion

4.1. Importance of AGE-antibodies in pathology and histochemistry

Many AGE antibodies currently used for immunohistochemical studies are raised by immunizing animals with

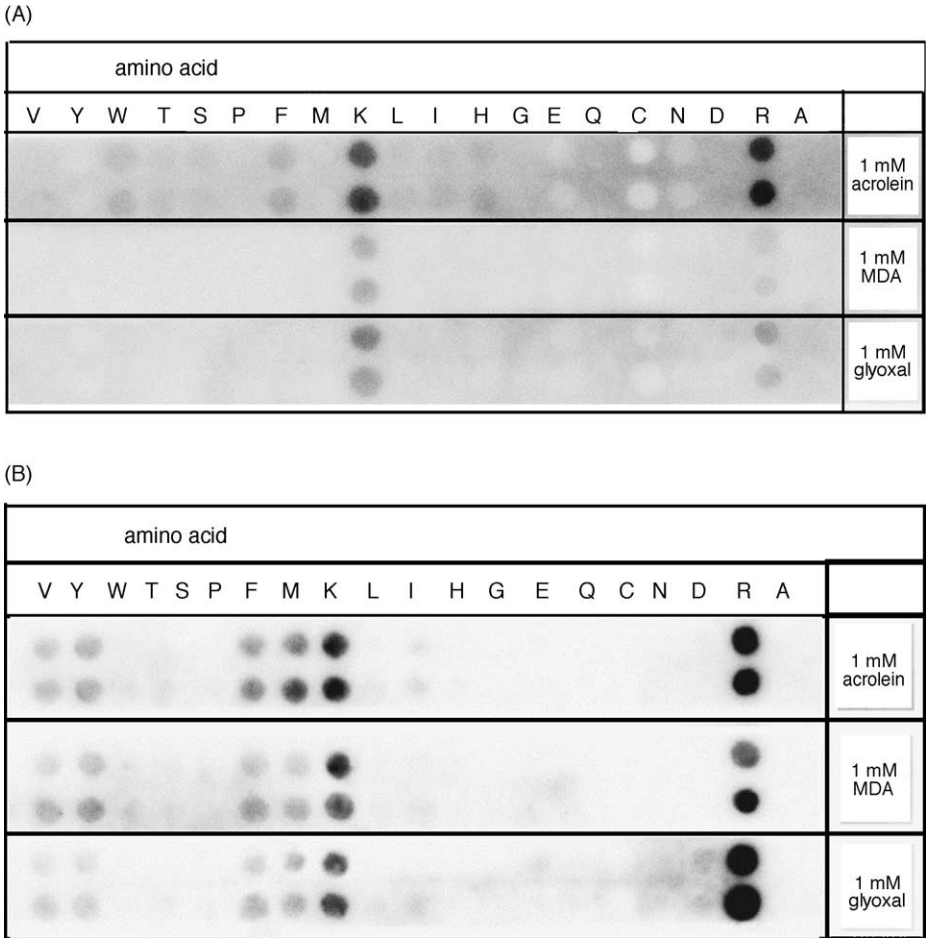

Fig. 5. Reaction of carbonyls with terminal amino acids on peptide membranes. Each strip contains two rows, which were incubated under identical conditions with acrolein, MDA and glyoxal. Immunostaining was performed with antibody 4G9 (A) or antibody K2189 (B).

AGE-modified proteins, which were produced by prolonged incubation with glucose. It was assumed that AGE immunohistochemistry reflect a certain chemical or state condition in which formation of AGE is favored. For example, an increase in glucose concentration is assumed to be responsible for increased AGE-levels in diabetic tissue [2,14]. As a second example, increased concentration of methylglyoxal, which is produced by non-enzymatic degradation of triosephosphates, is believed to lead to increased levels of intracellular AGEs [25].

4.2. Specificity of AGE-antibodies

Our experiments showed, that glyoxal-modified, and in some cases, MDA- or acrolein-modified proteins can be detected by AGE-antibodies, which have been previously assumed to recognize glucose-derived modifications only. MAP-tau appears to be particularly sensitive to immunodetectable carbonyl modification. Although the studies, performed by different groups, might be difficult to compare, BSA was less reactive (5 sites) than MAP-tau (13 sites in the 4-repeat isoform, Lys-87, 132, 150, 163, 174, 225, 234, 259, 280, 281, 347, 353, and 369) are modified [21,26]. Such an increased reactivity of MAP-tau might explain why this protein is prone to AGE-mediated crosslinking and tangle formation [12]. We hypothesized that the different reactivities among the proteins might be caused by a difference in the content of basic amino acids. However, MAP-tau does not contain exceptionally many arginine and lysine residues (Table 1). Thus, it is likely that the reactivity also depends on the local microenvironment within the protein structure, which influences the p*I* and the accessibility of these residues.

Very interestingly, there is some difference between the immunoreactivity of carbonyl-modified proteins and the amino acids on the spot membrane. For glyoxal-modifications, a similar strong immunoreactivity on proteins and amino acids was found. While acrolein-modifications were always detected on the monopeptide spots, they were only detected on MAP-tau, but not on the other proteins. The recognition of MDA-modifications also depends on the antibody used and the modified protein. While MDA-modifications of MAP-tau are well recognized, modified HSA is only recognized by the CML antibody

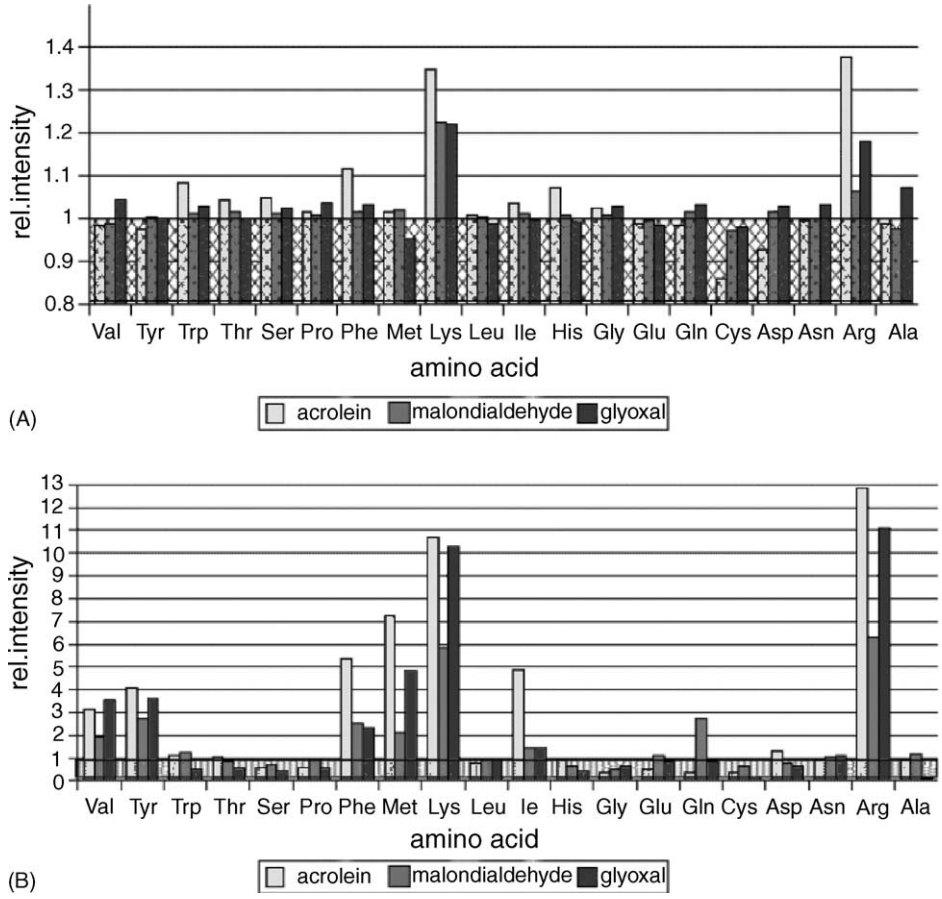

Fig. 6. Quantitative analysis of the immunoreactivity of the peptide spots modified with acrolein, MDA and glyoxal. To evaluate the antibody reactivity shown in Fig. 5, the relative intensity of the signal compared to the background (defined as 1.0) was calculated. Although the signal/noise ratio differs between the two antibodies, a significant immunoreactivity of lysine- and arginine-modifications can be observed for antibody 4G9 (A) and antibody K2198 (B).

4G9. One explanation for the difference between carbonyl modified proteins and amino acids could come from the difference in density of the reactive sites. Whereas the molar amount of protein on the nitrocellulose spots is in the picomolar range, it is in the nanomolar range with the immobilized amino acids (Table 2). The high density of the same amino acid on the spot membrane might allow bifunctional carbonyl compounds to form crosslinks (homodimers) between two adjacent amino acid residues. In the carbonyl-modified proteins, lysine or arginine residues are rarely in close proximity to other reactive sites, and are rather likely to form monomeric than dimeric AGE- or ALE structures. If these assumptions are correct, the antibodies (particularly in the case of acrolein) would preferentially bind to epitopes which are formed by two amino acids rather than one.

On the molecular level, the detection of glyoxal, acrolein or MDA-modified proteins by AGE-antibodies may be the result of either: (a) the formation of the identical compound from glyoxal, MDA or acrolein as from glucose (indicating a convergence of lipid and sugar chemistry) or (b) an immunological crossreactivity of the AGE-antibody with a structurally related compound formed from glyoxal, MDA or acrolein.

Table 1
Arginine and lysine content of the investigated proteins

Protein	Amino acids	Number of lysine residues + N-terminus	Percentage of lysine	Number of arginine residues	Percentage of arginine	Percentage of lysine + percentage of arginine
Chicken egg albumin	385	20 + 1	5.5	15	3.9	9.4
HSA	585	59 + 1	10.2	24	4.1	14.3
MAP-tau	757	64 + 1	8.6	30	4.0	12.6
β-Amyloid peptide (1–40)	40	2 + 1	7.5	1	2.4	9.9

Table 2
Molar amount of protein in each spot (per 30 ng of protein)

	AA	MW (g/mol)	Protein per protein spot (pmol)	Lysines per protein spot (pmol)	Arginines per protein spot (pmol)
Chicken egg albumin	385	42,350	0.71	14.2	10.6
HSA	585	64,350	0.47	27.5	11.2
MAP-tau	757	83,270	0.36	23.2	10.8
β-Amyloid peptide (1–40)	40	4,400	6.8	13.6	6.8
In comparison: amino acids on peptide spot membrane				17,000	17,000

4.2.1. Chemically identical structures

There is evidence from the literature that certain lysine-based AGE-structures can be formed from either glucose or glucose degradation products or from glyoxal, MDA or acrolein. Since glucose-modified proteins containing fructosyl-lysine degrade and yield glyoxal, methylglyoxal and 3-deoxyglucosone [29,34], the resulting endproducts are likely to be identical.

N(epsilon)-(carboxymethyl)lysine (CML) is one of these defined AGE-structures [35], and it was found that CML is also formed upon oxidation of low density lipoproteins what suggests that lipoxidation-derived carbonyl species are sources of CML [8,24]. Less is known about arginine-ALEs and their structural homology to glucose-derived arginine modifications. One well investigated structure is *N*(delta)-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1) which is formed from glucose as well from glucose degradation products [28].

4.2.2. Immunological crossreactivity of the AGE-antibody with a structurally related compound formed from glyoxal, MDA or acrolein

If immunoreactivity of AGE-antibodies with ALEs is not caused by the presence of identical antigenic compounds, immunological crossreactivity between AGEs and ALEs is the most likely alternative. Since AGEs are heterogeneous structures, antibodies (especially polyclonal antibodies such as K2189) are not expected to distinguish between them and related structures when their recognition pattern has not to be restricted on unique structural elements that would exclude a wide range of possible AGEs formed. Such a known related heterocyclic compound that is not formed with glucose but with a carbonyl (acrolein) may be *N*-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) or *N*(epsilon)-(3-methylpyridinium)lysine (Fig. 1D). These products are derived from the reaction between acrolein and lysine [9,32], and *N*(epsilon)-(3-methylpyridinium)lysine is most likely the main antigenic product on the peptide membranes on the 'lysine/acrolein' spot [9].

The polyclonal antibody K2189 shows immunoreactivity towards spots with planar cyclic amino acids such as tyrosine and phenylalanine but also valine and methionine. This crossreactivity appears to be rather an unspecific reactivity of the K 2189 antibody towards heterocyclic and/or small hydrophobic amino acids. We also observed an intense reactivity of the monoclonal 4G9 (CML) antibody with the carbonyl modified side-chains of arginine on the peptide membranes, indicating a previously unknown reactivity of this antibody in addition to the well known recognition of the CML epitope. Since the monoclonal 4G9 antibody was originally raised against KLH-AGE, and the positive clones screened for reactivity with CML, it could be possible that it reacts with a similar structure on the arginine side chain such as carboxymethylarginine (CMA) [10].

AGEs are considered to be endproducts of glycation or glycoxidation reactions, they therefore, should be formally distinguished from modifications with reactive carbonyl species. However, if AGEs such as CML or AGE-related structures such as FDP-lysine can be produced by lipid peroxidation reactions, then oxidative stress has to be considered to be an additional cause for formation of AGE-like epitopes with consequences on interpretation of previous histochemical work on AGEs.

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