Immunochemical detection of oxalate monoalkylamide, an ascorbate-derived Maillard reaction product in the human lens

Ramanakoppa H. Nagaraj[a,b], Farrukh A. Shamsi[a], Birgit Huber[c], Monika Pischetsrieder[c,*]

[a] *Center for Vision Research, Department of Ophthalmology, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, OH 44106, USA*
[b] *Institute of Pathology, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, OH 44106, USA*
[c] *Institute for Pharmacy and Chemistry, University of Munich, Munich, Germany*

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Abstract Carbohydrates with reactive aldehyde and ketone groups can undergo Maillard reactions with proteins to form advanced glycation end products. Oxalate monoalkylamide was identified as one of the advanced glycation end products formed from the Maillard reaction of ascorbate with proteins. In these experiments, we have analyzed human lens proteins immunochemically for the presence of oxalate monoalkylamide. Oxalate monoalkylamide was absent in most of the very young lenses but was present in old and cataractous lenses. The highest levels were found in senile brunescent lenses. Incubation experiments using bovine lens proteins revealed that oxalate monoalkylamide could form from the ascorbate degradation products, 2,3-diketogulonate and L-threose. These data provide the first evidence for oxalate monoalkylamide in vivo and suggest that ascorbate degradation and its binding to proteins are enhanced during lens aging and cataract formation.

Key words: Maillard reaction; Human lens; Oxalate monoalkylamide; Ascorbate degradation

1. Introduction

The human lens undergoes numerous physico-chemical changes during aging. The Maillard reaction, non-enzymatic condensation of sugars with proteins, has been considered as one of the mechanisms in lens aging. The end products of this reaction, referred to as advanced glycation products (AGEs), have been shown to accumulate with age in lens proteins [1]. In both senile and diabetic cataractous lenses, increased levels of AGEs have been observed [2,3]. A recent study has shown that cataracts can be prevented in mildly diabetic animals by inhibiting the Maillard reaction.

In the human lens, ascorbate (ASC) is present in relatively large amounts (up to 2 mM) [4] and has been shown to undergo oxidative degradation with age and cataract formation [5–9]. In vitro incubation experiments have revealed that the degradation products of ASC can rapidly react with lens proteins through Maillard reactions to form cross-linked proteins with characteristic browning and fluorescence. These changes resemble those occurring in the lens during normal aging and cataract formation. Several Maillard reaction products, pentosidine, carboxymethyllysine (CML) and LM-1 have been identified in the human lens and have been shown to form from ASC.

Previously, it was shown that oxalate monoalkylamide (OMA) is a major product in the Maillard reaction initiated by ASC [10]. Polyclonal antibodies were raised against this modification and it was determined that proteins undergoing Maillard reactions with ASC produce OMA. It was also shown that Maillard reaction products of sugars such as glucose are not recognized by the antibody, but more recent experiments reveal that they do produce OMA in long-term incubations, although at a much smaller amount when compared to ASC. Thus, ASC appears to be the major precursor of OMA [11]. In this report, we describe the occurrence of OMA in the human lens and its relation to lens aging and cataract formation.

2. Materials and methods

Human lenses were obtained from The Cleveland Eye Bank, Cleveland, The National Disease Research Interchange, Philadelphia and The University Hospitals of Cleveland. Bovine lenses were from Pel-Freeze Biologicals, Rogers (AR, USA). ASC and L-threose were obtained from Sigma Chemical (St. Louis, MO, USA). 2,3-Diketogulonate (DKG) was synthesized by the method of Otsuka et al [12]. The anti-OMA antibody was produced in rabbits [11] and the IgG fraction from the antiserum was purified on a protein-G column. BSA-OMA was prepared by the previously published method [11].

2.1. Human lens proteins

Each human lens was decapsulated and extracted by homogenization in 2 ml PBS, followed by centrifugation at 16 000×*g* for 30 min. The supernatant was dialyzed against 2 l PBS for 24 h, lyophilized and stored at −80°C until use. 61 μg protein was used from each sample for the competitive enzyme-linked immunosorbent assay (ELISA).

2.2. Incubation of bovine lens water-soluble proteins (BLP) with ASC and ASC oxidation products

Bovine lenses were extracted as described elsewhere [13]. 10 mg/ml water-soluble protein in 0.2 M sodium phosphate buffer (pH 7.4) was incubated with 100 mM of one of ASC, DKG or L-threose, for 2 weeks at 37°C and dialyzed against PBS for 48 h and lyophilized.

2.3. ELISA

The microplate wells were coated with 1 mg/well BSA-OMA in 0.02 M sodium carbonate buffer, pH 9.7, overnight at 4°C. The wells were washed 2× with PBS containing 0.1% Tween-20 (PBST) and blocked with 3% gelatin in PBST for 2 h at room temperature in a humid chamber. Following washing 3× with PBST, antibody (1:1000 dilution in PBST, IgG fraction from the antiserum purified on protein-G Sepharose) was added directly or pre-incubated (1 h at 37°C) with the test protein and incubated for 2 h. In the case of BLP incubation experiments, antiserum was directly used at a 1:10 000 dilution. The

*Corresponding author. Fax: (49) (89) 5902447.
E-mail: pischets@pharmchem.uni-muenchen.de

Abbreviations: OMA, oxalate monoalkylamide; ELISA, enzyme-linked immunosorbent assay; BLP, bovine lens protein; ASC, ascorbate; DKG, 2,3-diketogulonate; GSH, glutathione

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wells were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 2 h (1:60 000 dilution in PBST), washed four times with PBST and incubated with *p*-nitrophenylphosphate substrate at pH 9.8 until the *OD* at 406 nm reached about 0.6.

2.4. Western blotting

The water-soluble lens proteins (20 μg) from a young (16 years) and an old lens (65 years) were subjected to electrophoresis on a 10% SDS gel (60 min, 40 mA). The proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad laboratories, Richmond, CA, USA) for 2 h at 90 V. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline-Tween-20 (TBST) buffer, pH 7.0 (blotto), overnight at 4°C and then allowed to react for 2 h with the anti-OMA antibody (1:250 diluted in blotto). In an experiment to check the specificity of the reaction of anti-OMA antibody with lens proteins, the antibody was pre-incubated with 50 mg BSA-OMA at 37°C for 2 h and then at 4°C for 16 h prior to incubation with the membrane. The membrane was washed three times with TBST buffer and incubated with 1:3000 diluted anti-rabbit IgG coupled to horseradish peroxidase (Sigma) in blotto for 1 h, washed at least 8–10 times with TBST buffer and developed with the Supersignal chemiluminescence substrate kit (Pierce, Rockford, IL, USA) for 3–5 min and exposed to X-ray film (reflection/autoradiography film from NEN Life Science Products, Boston, MA, USA) in a dark room.

2.5. Statistical analysis

Differences between groups were analyzed by ANOVA using the Fisher PLSD test. $P < 0.05$ was considered significant.

3. Results

The IgG fraction was purified from OMA antiserum on a protein-G Sepharose column and used in a competitive ELISA to determine the presence of OMA in human lens proteins. Three representative lenses, young (30 years), cataractous (68 years) and brunescent (70 years), were used to test the effect of an increasing protein concentration on the immunoreactivity. A maximum of 5, 14 and 26% inhibition was observed for young, cataractous and brunescent lens proteins, respectively (Fig. 1). The percent inhibition corresponds to almost three and five times more antigen in the cataractous and the brunescent lens relative to the young lens.

The immunoreactivity in individual young (11–33 years), old (58–75 years), cataractous (59–85 years), diabetic (17–67 years, type II, non-cataractous) and brunescent lenses (60–80 years) was investigated. Most of the young lenses failed to show immunoreactivity (Fig. 2). Almost an equal immunoreactivity was observed for old and diabetic lenses. On the other hand, the mean level in cataractous lenses was about 1.5 times higher than the mean level in old lenses. Brunescent lenses showed significantly higher levels (percent inhibition: 30.35 ± 14.28; old 9.49 ± 7.99) when compared to the rest of the lenses ($P < 0.05$).

Western blotting was performed to further confirm the immunoreactivity in human lenses. The immunoreactivity was primarily localized to one of the protein bands in the high molecular weight region (Fig. 3) and the molecular weight of this band appears to be about 45 kDa. Diffused immuno-

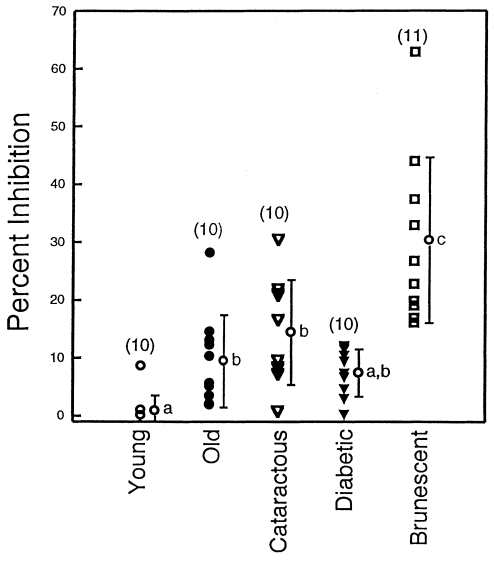

Fig. 2. The effect of age, diabetes and cataract on the immunoreactivity of human lens proteins against OMA antibody. 61 μg protein from each lens was tested in a competitive ELISA as described in Section 2.

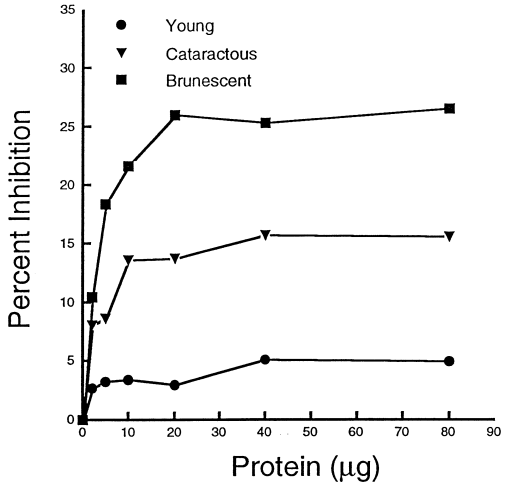

Fig. 1. The effect of an increasing lens protein concentration on the immunoreactivity against OMA antibody. Representative samples from young (30 years old), cataractous (68 years) and brunescent (70 years) lenses were used. Immunoreactivity was determined by a competitive ELISA.

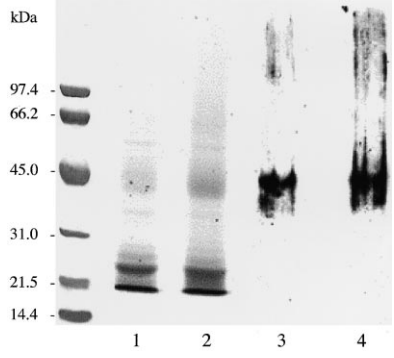

Fig. 3. Western blotting of human lens water-soluble proteins using OMA antibody. 10 μg protein from a young (16 years) and an old lens (65 years) was subjected to electrophoresis on a 10% SDS gel. The proteins were electrophoretically transferred to a nitrocellulose membrane and reacted with OMA antibody followed by goat anti-rabbit IgG coupled to horseradish peroxidase. The immunoreactivity was detected using the Pierce Supersignal substrate kit. Lanes 1 and 2 are from Coomassie blue-stained SDS-PAGE of 16 year and 65 year old lens proteins, lanes 3 and 4 are from corresponding Western blotting.

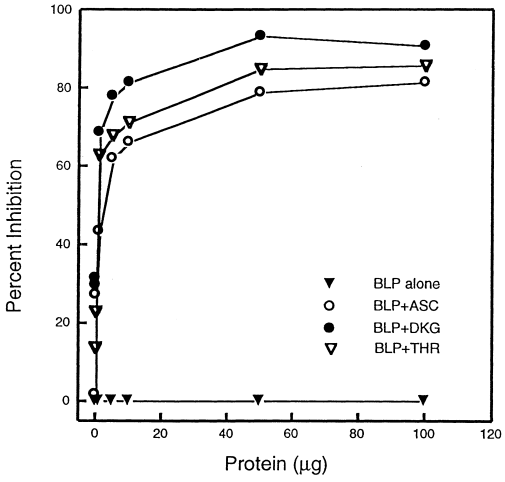

Fig. 4. Formation of OMA in water-soluble BLPs incubated with ASC and its degradation products. Lens proteins (10 mg/ml) in 0.2 M phosphate buffer were incubated with 100 mM indicated carbohydrate, dialyzed and used in a competitive ELISA.

reactivity could be seen in other higher molecular weight proteins. The protein band of 45 kDa could be a dimer of crystallins, whereas the diffused streaks at the high molecular region are probably from highly cross-linked proteins that occur during aging and also during the Maillard reaction [14]. Thus, it can be concluded that formation of OMA in vivo occurs concurrently with protein cross-linking. The immunoreactivity could be completely abolished by pre-incubation of the antibody with BSA-OMA. Furthermore, normal rabbit IgG at the same concentration as OMA-IgG failed to show immunoreactivity (data not shown). These results suggest that the antibody specifically reacted with OMA, present in lens proteins.

To determine the formation of OMA from ASC and its degradation products, incubation experiments were performed. Water-soluble BLPs were incubated at physiological conditions with ASC and its degradation products, DKG and

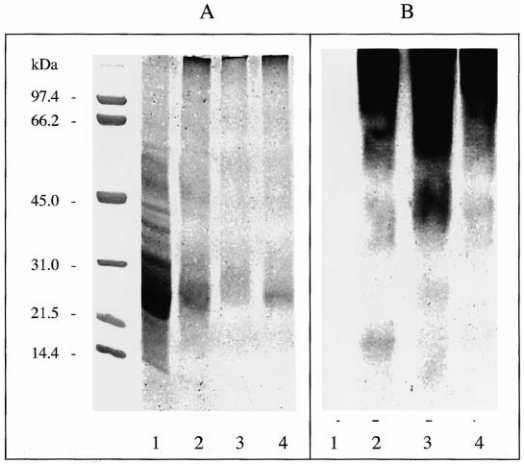

Fig. 5. Western blotting of BLPs modified by ASC and its degradation products. A: Coomassie blue-stained SDS-PAGE gel and B: Western blotting using antiserum against OMA. Lanes 1, BLP incubated without carbohydrate; lanes 2, BLP+ASC; lanes 3, BLP+DKG and lanes 4, BLP+threose. Other details are the same as in Fig. 3.

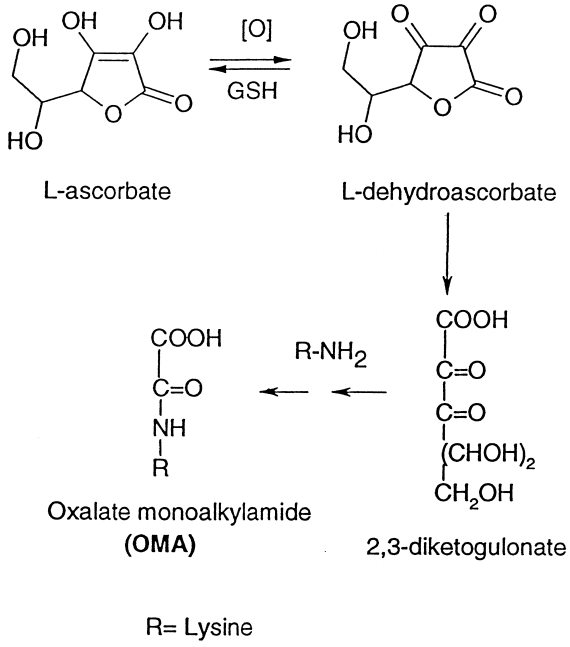

Fig. 6. Possible pathway of OMA formation from ASC. ASC must undergo oxidation to form OMA in proteins.

L-threose, and tested for immunoreactivity using antiserum against OMA. OMA synthesis occurred during all three incubations. A slightly higher immunoreactivity is detectable in the samples that were incubated with DKG compared to those with L-threose or ASC (Fig. 4). The relative inhibition was 66.1, 81.3 and 71.1 for 10 mg protein incubated with ASC, DKG and L-threose, respectively.

The Western blotting results (Fig. 5) showed a diffused immunoreactivity in high molecular weight proteins. The immunoreactivity of the native protein was negligible as opposed to the extensive immunoreactivity in the high molecular weight region in ASC-modified proteins. This suggests that incubation of proteins with ASC and its oxidation products leads to extensive cross-linking and formation of high molecular weight protein aggregates that do not migrate well in the gel but contain significant amounts of OMA. The protein band of 45 kDa present in the human lens was not apparent in ASC-modified bovine proteins possibly because of its further modification and/or cross-linking with other proteins in the high molecular weight region. Although OMA does not cross-link proteins, its presence in high molecular weight proteins suggests that its formation occurs concurrently to cross-linking AGEs. Taken together, these results suggest that DKG and other degradation products of ASC are likely to be the major source of OMA in the lens.

A possible pathway of OMA synthesis is shown in Fig. 6. A possible mechanism is that DKG first gives rise to a Schiff's base which then degrades to produce OMA and threonic acid. This is based on the reported formation of oxalate from DKG [15]. The reaction pathway of OMA formation from threose is unclear, but it is obvious that several oxidation steps are involved. Oxidation reactions of the Amadori product of L-threose and other α-amino-ketones are very likely, because it has been shown that structures of this type are spontaneously oxidized by oxygen [16,17]. Retro-aldol cleavage and further oxidation would then lead to the formation of OMA.

4. Discussion

Several previous independent studies have suggested that human lens proteins may undergo Maillard reactions by ASC oxidation products during aging and cataractogenesis. Although no direct evidence is available, results from in vitro incubation experiments,and detection of Maillard products that could be synthesized from ASC oxidation products provided support for this assumption. The present study establishes, for the first time, that ASC-mediated Maillard reactions occur in the human lens and may play a role in protein modifications during aging and cataract formation.

For the synthesis of OMA, ASC must be oxidized before the oxidation products react with protein lysine residues. ASC is present in relatively large amounts in the human lens, possibly to protect lens proteins from oxidative damage. In the lens, glutathione (GSH) and enzymes maintain ASC in its reduced state [18–20]. However, during aging and cataract formation, the lens GSH level is severely depleted which could lead to an enhanced oxidation of ASC. The detection of increasing amounts of DHA and 2,3-DKG in relation to the severity of cataract [5] supports this view. Additionally, calcium oxalate, which may originate from ASC oxidation [21] was found in cataractous lenses. It was reported that there is a considerable decrease in the amount of ASC in brunescent lenses, suggesting its accelerated oxidation [18]. The finding in the present study that brunescent lenses contain the highest immunoreactivity for the OMA antibody supports an enhanced ASC oxidation in these lenses.

Diabetic lenses were expected to have a higher OMA immunoreactivity than age-matched normal lenses, because diabetes, at least in animals, results in a drastic depletion of GSH in the lens. This could lead to oxidation of ASC and formation of OMA. The following observations support this view. Experimental diabetes in rats causes a decrease in reduced ASC [22] and in organ-cultured galactosemic rat lenses, an increase of the ASC-mediated glycation has been observed [23], suggesting an enhanced ASC oxidation and Maillard reactions during hyperglycemia. However, we found almost identical levels of OMA immunoreactivity in diabetic and non-diabetic lenses. The diabetic lenses that we used were non-cataractous and therefore, the extent of ASC oxidation may not have been significantly different when compared to age-matched old lenses. Experimental diabetic cataract may be useful in understanding ASC oxidation, OMA formation and their relation to lens GSH.

In summary, this study provides evidence for OMA in the human lens. OMA is most likely derived from ASC-mediated Maillard reactions. The relevance of OMA to lens aging and development of cataract remains to be studied.

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