SCIENTIFIC REPORT

Advanced glycation end product deposits in climatic droplet keratopathy

Y Kaji, R Nagai, S Amano, Y Takazawa, M Fukayama, T Oshika

Br J Ophthalmol 2007;91:85–88. doi: 10.1136/bjo.2006.099812

Background: Climatic droplet keratopathy (CDK), known as spheroid degeneration of the cornea, is one of the most frequent degenerative corneal disorders affecting visual function. However, the histochemical nature of the deposits seen in CDK is still unclear.

Aim: To investigate the pathogenesis of CDK, we investigated the immunohistochemical localisation of advanced glycation end products (AGEs) in surgical specimens of CDK.

Methods: Immunohistochemical localisation of N^ε-(carboxymethyl)-L-lysine (CML), N^ε-(carboxyethyl)-L-lysine (CEL), pyrrolidine, pentosidine and imidazolone was examined in three corneas with CDK, six corneas with bullous keratopathy and three corneas without any corneal diseases.

Results: In all the specimens with CDK, immunoreactivity was strong in CML, moderate in pyrrolidine and pentosidine, and weak in imidazolone. Immunoreactivity was absent in CEL. In contrast, no immunoreactivity to CML, pyrrolidine, pentosidine, imidazolone or CEL was detected in corneas with bullous keratopathy, or in corneas without any corneal diseases.

Conclusions: CDK is caused by an aggregation of AGE-modified proteins. The result is consistent with etiological findings that ultraviolet irradiation and ageing, both of which are accelerators of AGE formation, are closely related to the development of CDK.

Climatic droplet keratopathy (CDK), also known as spheroidal degeneration of the cornea, is one of the most frequent degenerative corneal diseases with high prevalence rates especially in equatorial and polar regions.¹ Clinically, CDK is observed as yellowish deposits in the superficial cornea, especially in the interpalpebral area. Histologically, numerous deposits of eosinophilic amorphous bodies are observed in Bowman's layer and the superficial layer of the corneal stroma in CDK. Although a number of studies have investigated the deposits, their histochemical nature is still unclear.

Epidemiological studies have shown that ultraviolet rays and the ageing process are closely related to the pathogenesis of CDK.^{1,2} For example, a high prevalence rate of CDK has been reported in the Red Sea region, Africa and Labrador, where intense ultraviolet irradiation occurs.¹ In addition, the prevalence rate of CDK increases with age in all parts of the world. For this reason, the pathogenesis of CDK is considered to be closely related to both ultraviolet irradiation and the ageing process.

Advanced glycation end products (AGEs) are the final reaction products of sugars and proteins and are closely related to ultraviolet irradiation and the ageing process. AGE formation progresses very slowly because of the non-involvement of enzymes in the process. For this reason, AGEs are detected in the body in areas with low turnover rates, such as dura mater, lenses and basement membranes. Furthermore, ultraviolet

irradiation is known to accelerate the formation of AGEs which therefore accumulate in the bodies of elderly people. Conversely, AGE deposits accelerate the ageing process because they induce oxidative stress and molecular damage closely related to the ageing process. For this reason, it was hypothesised that CDK is caused by the accumulation of AGE deposits. To confirm this hypothesis, the immunohistochemical localisation of AGEs was investigated in surgical specimens with CDK.

MATERIALS AND METHODS

Surgical specimens of cornea with or without climatic droplet keratopathy

Informed consent was given by all the patients undergoing corneal surgery in the present study. Three corneas with CDK were obtained from three patients (two men and one woman, mean (standard deviation (SD)) age 62 (4) years) during penetrating keratoplasty. One of the male patients, an outdoor worker, had no history of keratitis, indicating that he had primary CDK. The other two patients had histories of interstitial keratitis with congenital syphilis, indicating that they had secondary CDK. In addition, six corneas with bullous keratopathy from six patients (four men and two women, mean (SD) age 58.3 (8.4) years) and three corneas without any corneal diseases from three patients (two men and one woman, mean (SD) age 66 (6.2) years) were obtained. Haematoxylin-eosin staining and immunohistochemistry, described below, were performed on all surgical specimens.

Monoclonal antibody to CML and CEL

Monoclonal antibody to N^ε-(carboxymethyl)-L-lysine (CML) or N^ε-(carboxyethyl)-L-lysine (CEL) was prepared as described previously.³ Briefly, BALB/c mice were injected with CML-bovine serum albumin (BSA) or CEL-BSA in 50% Freund's complete adjuvant. Then, the splenic lymphocytes from the immunised mice were fused to myeloma P3U1 cells in the presence of polyethylene glycol. One cell line positive for CML-BSA or CEL-BSA was selected through successive subcloning. This cell line was inoculated into the peritoneal cavities of BALB/c mice and antibodies were purified from ascetic fluid by protein G affinity chromatography to immunoglobulin(Ig)G₁.

Monoclonal antibodies to pyrrolidine and pentosidine

Monoclonal antibodies to pentosidine and pyrrolidine, N,N-diacetyl-pentosidine and caproyl pyrrolidine, were prepared as described previously.⁴ Splenic lymphocytes from BALB/c mice immunised with N,N-diacetyl-pentosidine-conjugated keyhole limpet haemocyanin (KLH) were fused to myeloma P3U1 cells. One cell line, termed 4D7, was produced from the ascitic fluid of BALB/c mice bearing the hybridoma cells, and further

Abbreviations: AGE, advanced glycation end product; BSA, bovine serum albumin; CDK, climatic droplet keratopathy; CEL, N^ε-(carboxyethyl)-L-lysine; CML, N^ε-(carboxymethyl)-L-lysine; HSA, human serum albumin; KLH, keyhole limpet haemocyanin

Figure 1 Immunohistochemical localisation of advanced glycation end products. Eosinophilic and amorphous materials are observed in the superficial layer of the corneal stroma (A) in case 1 with climatic droplet keratopathy. The materials were positive for N^ε-(carboxymethyl)-L-lysine (B), pyrraline (C), pentosidine (D) and imidazolone (E). In contrast, the immunoreactivity to N^ε-(carboxyethyl)-L-lysine was not detected in CDK (F). No staining was detected in negative control (G).

purified by protein G affinity chromatography to IgG₁. The monoclonal antibody to pyrraline was prepared in a similar way by immunising caproyl pyrraline-conjugated human serum albumin (HSA).

Monoclonal antibody to imidazolone

To prepare monoclonal antibody to N^ε-benzyloxycarbonyl-imidazolone (imidazolone), 6 mg of the synthesised imidazolone was incubated with 6 mg of HSA. Then the splenic lymphocytes from BALB/c mice immunised with imidazolone-HSA were fused to myeloma P3U1 cells. The hybridoma cells positive for imidazolone-conjugated KLH but negative for HSA and KLH were selected through successive subcloning. Monoclonal antibodies to imidazolone-HSA were further purified by protein G-immobilised Sepharose gel to IgG₁.

Immunohistochemistry

Multiple 3-μm-thick sections of formalin (10%)-fixed, paraffin-embedded corneal specimens were prepared. The immunohistochemical localisation of CML, CEL, pyrraline, pentosidine and imidazolone was examined using a streptavidin-biotin complex method (SAB-PO(M) Kit, Nichirei, Tokyo, Japan) and the monoclonal antibodies were prepared as above. The final reactive products were visualised by 3,3'-diaminobenzidine (Sigma). Negative controls were prepared by replacing monoclonal antibodies to AGE with mouse non-immunised IgG₁

(Sigma). The nuclei were counterstained with Mayer's haematoxylin solution. The experiments were repeated three times in each surgical specimen.

Table 1 summarises the results of the immunohistochemical examination.

RESULTS

Figure 1 shows examples of haematoxylin-eosin staining and immunohistochemistry in cornea with primary CDK. In CDK specimens, deposits of eosinophilic and amorphous materials were observed in the superficial layer of the corneal stroma (fig 1A). The corneal epithelium was atrophic and partly detached from the corneal stroma. Strong immunoreactivity to CML was detected in the amorphous materials in the superficial layer of the corneal stroma (fig 1B). Moderate immunoreactivity to pyrraline (fig 1C) and pentosidine (fig 1D), and weak immunoreactivity to imidazolone (fig 1E) was also detected in the amorphous materials in CDK specimens. In contrast, immunoreactivity to CEL was not detected in any specimens with CDK (fig 1F). No staining was detected in the negative control (fig 1G). The result of the immunohistochemistry was almost the same as that of primary CDK. The corneal epithelium was atrophic without detachment from the corneal stroma.

In corneal specimens with band keratopathy and bullous keratopathy, and in corneas without any corneal diseases, no

Table 1 Results of immunohistochemical examination

	Number	Sex	Age	Immunohistochemistry				
				CML	Pyrraline	Pentosidine	Imidazolone	CEL
CDK	1	Male	66	++	+	+	±	—
	2	Male	62	++	+	+	±	—
	3	Female	58	++	+	±	±	—
Bullous keratopathy	4	Male	71	—	—	—	—	—
	5	Female	62	—	—	—	—	—
	6	Male	61	—	—	—	—	—
	7	Male	58	—	—	—	—	—
	8	Male	49	—	—	—	—	—
	9	Female	49	—	—	—	—	—
Control	10	Male	71	—	—	—	—	—
	11	Male	68	—	—	—	—	—
	12	Female	59	—	—	—	—	—

immunoreactivity to CML, CEL, pyrraline, pentosidine or imidazolone was detected.

DISCUSSION

To examine the pathogenesis of CDK, the immunohistochemical localisation of AGEs was investigated in surgical specimens with CDK. The results showed that immunoreactivity to CML, pyrraline, pentosidine and imidazolone was detected in the deposits in all specimens with CDK. In contrast, none of the AGE components were detected in the corneal stroma without CDK. The results indicate that the pathogenesis of CDK is related to the aggregation and accumulation of AGEs in the cornea.

The results of this study agree with the results of the previous study on CDK as follows. Firstly, the deposits in CDK are extremely insoluble. This insoluble property is one of the common characteristics of AGE-modified proteins where intramolecular and intermolecular crosslinkings are often seen. Secondly, amino acid analysis of the deposits seen in CDK could not show their nature or origin. Any intracellular or extracellular protein in the body is capable of being AGE modified. This means that the deposits in CDK would be aggregations of various AGE-modified proteins. For this reason, amino acid analysis could not be a suitable method for examining the nature of the deposits in CDK.

In aged patients or those with diabetes, AGEs tend to accumulate in pinguecula,⁵ cataracts,⁶ drusen in the retina,⁷ lamina cribrosa,⁸ corneal endothelium,⁹ and basement membranes of the corneal epithelium.⁹ Many clinicopathological similarities exist between pinguecula and CDK. For example, aetiological studies have confirmed that ageing and ultraviolet irradiation are accelerators of both pinguecula and CDK. In addition, histological examinations have shown deposits of amorphous materials beneath the epithelial layer, known as elastotic degeneration, in both conditions.¹⁰ However, the pathogenesis of pinguecula and CDK had been unclear until now. We have recently shown that the pinguecula is caused by the deposition of AGEs including CML, pentosidine and pyrraline.⁵ Combined with the results of this study, the development of pinguecula and CDK are shown to have the same mechanism in common—that is, the deposition of AGEs. These results are consistent with the previous papers showing that the histochemical staining characteristics termed “elastotic degeneration” are similar in both pinguecula and CDK.

CML and CEL are quite alike in structure, however, the intensity of immunohistochemistry in CDK was quite different: strong in CML but none in CEL. The difference in the intensity of immunohistochemistry may be attributed to the origin of

CML and CEL. Ahmet *et al*¹¹ showed that aldose sugars such as glucose and ribose yield more CML than CEL. In contrast, methylglyoxal, a intermediate product of glucose and threonine, is a rich source of CEL.¹¹ For this reason, we speculate that the reaction of aldose sugars with lysine is involved in the development of CDK.

The prevalence of CDK is high especially in equatorial and arctic areas¹ and in the Dahlak Islands in the Red Sea, where CDK accounts for 57% of all cases of blindness.¹⁰ For this reason, the prevention and treatment of CDK is important for public health. The results of the present study indicate that inhibiting AGE formation will be a way to prevent CDK. Various factors are known to accelerate AGE formation, including ultraviolet irradiation, reactive oxygen species, increased concentration of sugars and high temperature. From the above, it can be concluded that protection from ultraviolet irradiation is the most practical way to prevent CDK. For this reason, ultraviolet-filtering sunglasses are recommended in outdoor workers in equatorial and arctic regions to reduce the number of people with blindness caused by CDK.

Authors' affiliations

Y Kaji, T Oshika, Department of Ophthalmology, Tsukuba University Institute of Clinical Medicine, Ibaraki, Japan

R Nagai, Department of Medical Biochemistry, Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

S Amano, Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

Y Takazawa, M Fukayama, Department of Pathology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

The Ministry of Education, Science, Sports and Culture (Grant-in-Aid for Young Scientists, 18791259, 2006) Japan supports this work.

Competing interests: None declared.

Correspondence to: Dr Y Kaji, Department of Ophthalmology, Tsukuba University Institute of Clinical Medicine, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8575, Japan; sanken-iky@umin.ac.jp

Accepted 1 August 2006

Published Online First 13 September 2006

REFERENCES

- Gray RH, Johnson GJ, Freedman A. Climatic droplet keratopathy. *Surv Ophthalmol* 1992;**36**:241–53.
- Dolin PJ, Johnson GJ. Solar ultraviolet radiation and ocular disease: a review of the epidemiological and experimental evidence. *Ophthalmic Epidemiol* 1994;**1**:155–64.

- 3 **Kume S**, Takeya M, Mori T, *et al*. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Am J Pathol* 1995;**147**:654–67.
- 4 **Hayase F**, Nagaraj RH, Miyata S, *et al*. Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction in vivo. *J Biol Chem* 1989;**264**:3758–64.
- 5 **Kaji Y**, Oshika T, Amano S, *et al*. Immunohistochemical localization of advanced glycation end products in pinguecula. *Graefe's Arch Clin Exp Ophthalmol* 2006;**244**:104–8.
- 6 **Monnier VM**, Cerami A. Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science* 1981;**211**:491–3.
- 7 **Ishibashi T**, Murata T, Hangai M, *et al*. Advanced glycation end products in age-related macular degeneration. *Arch Ophthalmol* 1998;**116**:1629–32.
- 8 **Amano S**, Kaji Y, Oshika T, *et al*. Advanced glycation end products in human optic nerve head. *Br J Ophthalmol* 2001;**85**:52–5.
- 9 **Kaji Y**, Usui T, Oshika T, *et al*. Advanced glycation end products in diabetic corneas. *Invest Ophthalmol Vis Sci* 2000;**41**:362–8.
- 10 **Rodrigues MM**, Laibson PR, Weinreb S. Corneal elastosis. Appearance of band-like keratopathy and spheroidal degeneration. *Arch Ophthalmol* 1975;**93**:111–14.
- 11 **Ahmed MU**, Brinkmann FE, Degenhardt TP, *et al*. N^ε-(carboxyethyl) lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 1997;**324**:565–70.