BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **226,** 37–41 (1996)
ARTICLE NO. 1308

Formation of Crossline as a Fluorescent Advanced Glycation End Product *in Vitro* and *in Vivo*

Hiroshi Obayashi,*,†,‡ Koji Nakano,†,[1] Hirofumi Shigeta,† Makiko Yamaguchi,† Kuniaki Yoshimori,† Michiaki Fukui,† Mitsuhiro Fujii,† Yoshihiro Kitagawa,† Naoto Nakamura,† Ko Nakamura,§ Yoshitaka Nakazawa,§ Kazuharu Ienaga,§ Mitsuhiro Ohta,‡ Masataka Nishimura,‡ Iwao Fukui,* and Motoharu Kondo†

**Department of Clinical Research, Kyoto Microbiological Institute, Kyoto, Japan; †The First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kajii-cho, Kawaramachidouri, Hirokojiagaru, Kamigyo-ku, Kyoto 602, Japan; §Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Co. Ltd. Osaka, Japan; and ‡Clinical Research Center, Utano National Hospital, Japan*

Received July 29, 1996

Crossline is one of the major advanced glycation end products resulting the reaction mixture of free amino group(s) such as ϵ-one in lysine with D-glucose *in vitro.* To study crossline formation on proteins *in vitro* and *in vivo,* polyclonal antiserum to the crossline hapten was prepared. This antiserum reacted with bovine and human serum albumin that had been modified by prolonged incubation with glucose as well as with crossline itself. Antisera did not react with unmodified serum albumin or the other Maillard-related compounds. Crossline was formed in a time-dependent manner when a mixture of six different proteins was incubated with glucose at pH 7.2 or 9.0. Crossline levels could be measured in rat lens proteins and the levels increased with age. The crossline content of lens proteins in diabetic rats was more than two-fold higher than that of age-matched controls. Results of this study suggest that most proteins containing advanced glycation end products have crossline-like structures. Measurement of crossline-like structures in biological specimens may provide an index of aging and of the development of diabetic complications.

Incubating proteins with reducing sugars such as glucose leads, through the early products such as Schiff's bases and Amadori adducts, to advanced glycation end products (AGEs) with fluorescence, brown color and cross-linking (1, 2). The characteristic fluorescence of AGEs (Ex 370/Em 440 nm) has been widely used as an indicator of the level of AGE-modified proteins. However, the structure of AGE fluorophores is unknown. The first putative fluorescent AGE, 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (FFI) (3) proved to be an artifact (4, 5). To date, the characterized fluorophores proven to be both a crosslink and an AGE are limited such as pentosidine (6) and crossline (7). However, the fluorescence spectrum of pentosidine (Ex 335/Em 385 nm) is substantially different from that of AGE-proteins *in vivo.* Crossline (Ex 379/Em 463 nm) is the first AGE-candidates having fluorescence characteristic similar to *in vivo* products. In the present study, formation of crossline-like structures *in vitro* and *in vivo* were investigated using specific antibodies to the crossline hapten.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (BSA), human serum albumin (HSA) and keyhole limpet hemocyanin (KLH) were purchased from Sigma (St. Louis, MO). A low molecular weight protein calibration (LMW) kit was purchased

[1] To whom correspondence should be addressed. Fax. 81-75-252-3721.

Abbreviations: AGE(s), advanced glycation end product(s); BSA, bovine serum albumin; HSA, human serum albumin; KLH, keyhole limpet hemocyanin; HPO, horseradish peroxidase; STZ, streptozotosin; PBS, phosphate buffered saline; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

0006-291X/96 $18.00

from Pharmacia (Uppsala, Sweden). Horseradish peroxidase (HPO)-conjugated anti-rabbit IgG was purchased from Bio-Rad (Richmond, CA). *N*-acetyllysine crossline was prepared as reported previously (7), pentosidine was prepared according to Shell and Monnier (6), caproylpyrraline was prepared according to Hayase *et al.* (8), calboxymethyllysine was prepared according to Dunn *et al.* (9) and *N*-Boc-hexitoyllysine was prepared according to Njoroge *et al.* (5).

Preparation of AGE-proteins. AGE-BSA and AGE-HSA were prepared by incubating BSA or HSA (50 mg/ml) with 0.5 M glucose in 0.2 M sodium phosphate buffer (pH 7.4) at 37 ℃ for 90 days under sterile conditions followed by exhaustive dialysis against deionized water. For in vitro time-course studies, one LMW vial was dissolved in 100 μl of 0.2 M sodium phosphate buffer (pH 5.0, 7.2 or 9.0) with or without 0.5 M glucose. Each sample was incubated at 37 ℃ under sterile conditions for 2, 4, 8 or 10 weeks and assayed by Western blot and ELISA.

Animals and preparation of lens proteins. Maile Wister rats aged 8 weeks were made diabetic by i.v. injection of streptozotocin (STZ: 60 mg/kg; Upjohn, Kalamazo, MI) in citrate buffer (pH 4.5). Animals with blood glucose $>$19.4 mmol/L at one week after injection were selected and killed at 12 or 20 weeks of age. Ten day-old rats and non-diabetic age-matched rats were used as controls. The lens was decapsulated and homogenized in 1.0 ml of phosphate buffered saline (PBS; pH 7.4) and centrifuged at 10,000 $\times g$ for 20 min at 4 ℃. The supernatant was used for assays. The protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

Preparation of anti-crossline antiserum. A one-to-one mixture (total 10 mg) of two *N*-acetyllysine-derived crossline epimers was conjugated with KLH (20 mg) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (67 mg; Kishida Chemical, Tokyo, Japan) in dimethyl formamide (3 ml; Wako Chemical, Osaka, Japan) and PBS (5 ml). After stirring at 4 ℃ overnight, the mixture was dialyzed in PBS and diluted to 15 ml with saline. An aliquot of the solution was mixed with the same volume of complete Freund's adjuvant and one third of the resulting emulsion was inoculated subcutaneously into each of three rabbits. The animals were boosted 14 days later with a one-to-one emulsion of antigen solution with incomplete Freund's adjuvant. After eight such boosters at 14 days intervals, antiserum was obtained.

Coupling of ϵ-aminocaproate-derived crossline to BSA. ϵ-aminocaproate crossline was purified from the reaction mixture resulting from incubation of glucose with ϵ-aminocaproate (Wako Chemical) at 37 ℃ for 4 weeks in sodium phosphate buffer (pH 7.4). ϵ-aminocaproate crossline (4.0 mg) and *N*-hydroxysulfosuccinimide (Pierce) were dissolved in 2.0 ml of ice cold water. To this solution was added 2.3 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. After 30 min at 0 ℃, BSA (50 mg) was added and stirred at room temperature for 18 h. The resulting solution was dialyzed against two liters of water for 24 h. Final concentration of BSA was quantitated to 8.5 mg/ml by BCA protein assay. Coupled ϵ-aminocaproate crossline was quantitated to 417 μg/ml using specific fluorescence (Ex 380/Em 460 nm). Approximately 6.2 moles of ϵ-aminocaproate crossline was incorporated onto 1 mole of BSA.

ELISA. Assay of crossline, ligand inhibition testing with various Maillard-related compounds and quantitation of crossline-bound protein were performed by competitive ELISA using 96-well ELISA plate at room temperature. Each well was coated with 100 μl of 10 μg/ml crossline-coupled BSA and blocked with 4% milk protein (Dai-Nippon Pharmaceutical, Osaka, Japan). After washing with PBS containing 0.02% Tween 20 (buffer A), to each well was added 50 μl of sample and 50 μl of anti-crossline antiserum diluted with 0.4% milk protein (final dilution, 1/2000) and the plate was incubated for 2 h. After washing with buffer A, 100 μl of HPO-conjugated anti-rabbit IgG (1:2000) was added to each well and the plate was incubated for 2 h. Plates were washed 5 times with buffer A and incubated with 100 μl of 0.04% o-phenylendiamine dihydrochloride (Wako Chemical) and 0.006% H2O2 in phosphate-citrate buffer (pH 5.0) as the enzyme substrate. The reaction was stopped by addition of 100 μl of 1M H2SO4 and the color reaction was read at 492 nm with an ELISA plate reader (Titertec Maltiskan MCC).

SDS-PAGE and Western blot. SDS-PAGE was performed according to the procedure of Laemmli on 4-20% gradient slab gels (10). One of each set of duplicate gels was stained for protein with Commassie blue and the other was electrophoretically transferred to a nitrocellulose membrane for immunoblotting according to the procedure of Towbin *et al.* (11). After transfer, membranes were blocked by incubating for 1 h in 4% milk protein at room temperature. After washing for 5 min with buffer A, membranes were incubated for 2 h in anti-crossline antiserum (1:500) at room temperature, washed with buffer A, incubated in HPO-conjugated anti-rabbit IgG (1:1000) for 2 h at room temperature, and washed with buffer A. The reaction products were visualized using 4-chloro-1-naphthol (4-CN; Wako Chemical) substrate (0.05% 4-CN and 0.01% H2O2 in PBS, pH 7.4).

RESULTS AND DISCUSSION

Characterization of anti-crossline antiserum. The immunoreaction of anti-crossline antibodies with crossline-coupled BSA in the competitive ELISA was inhibited by crossline, AGE-BSA and AGE-HSA but not by unmodified serum albumins or Maillard-related compounds such as pentosidine, caproylpyrraline, carboxymethyllysine or *N*-Boc-hexitoyllysine (Fig. 1). Such immunoreactivity was also observed in immunoblot analysis (Fig. 2). In addition, treatment of AGE-BSA with NaBH4 (30 min at 37 ℃) had no effect on the immunoreactivity

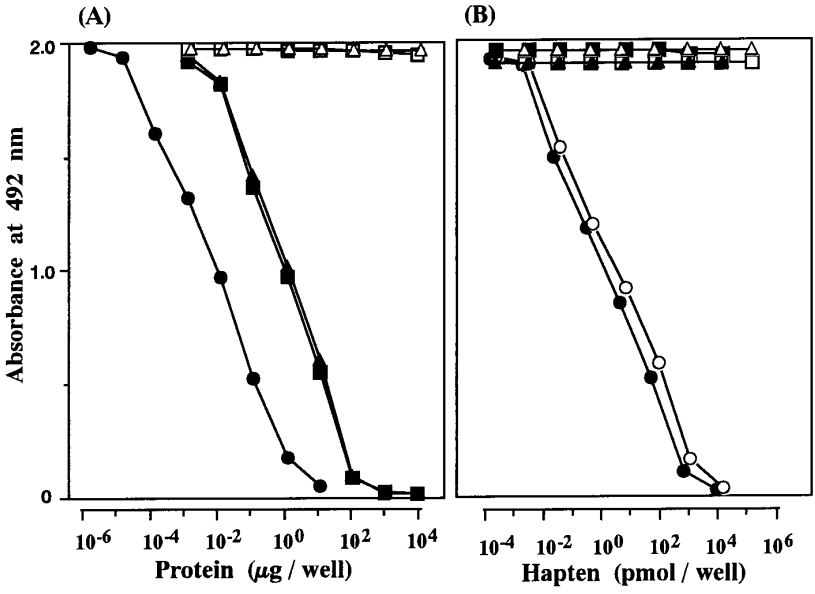

FIG. 1. ELISA inhibition curves. (A): ●, Crossline-coupled BSA; ■, AGE-BSA; ▲, AGE-HSA; □, BSA; △, HSA. (B): Maillard compounds: ●, *N*-acethyllysine crossline; ○, ϵ-aminocaproate crossline; ■, *N*-Boc-hexitoyllysine; □, pentosidine; ▲, caproylpyrraline; △, carboxylmethyllysine.

(data not shown). These results indicated that the antiserum was specific to the crossline hapten and that AGE-proteins formed in vitro indeed contain crossline-like structure(s).

Time course of crossline formation. SDS-PAGE analysis (Fig. 2A) revealed shifts lower apparent molecular weights and broadening of the protein-staining bands over time in the

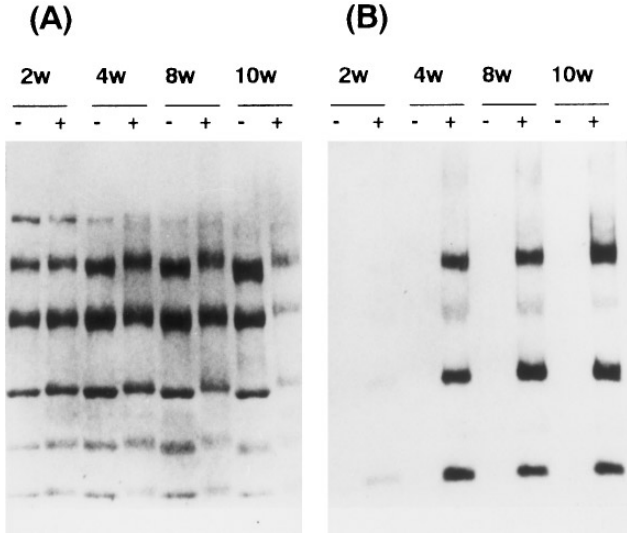

FIG. 2. SDS-PAGE profile (A) and Western blot analysis with anti-crossline (B). Sample proteins incubated with (+) or without (−) glucose for the number of weeks indicated.

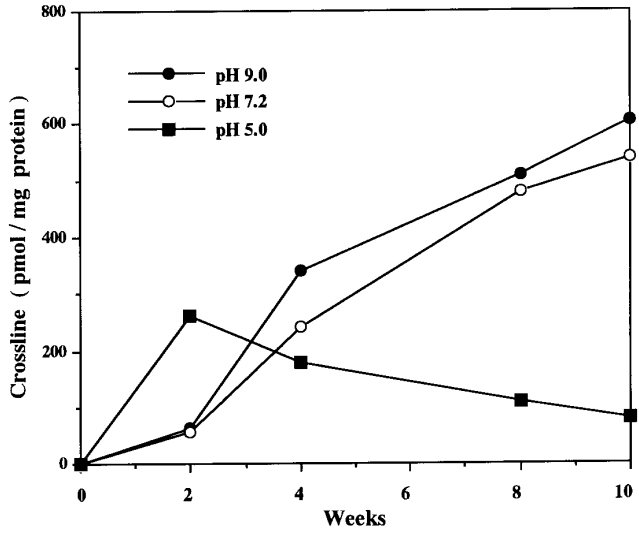

FIG. 3. The effect of pH on crossline formation by competitive ELISA.

absence of glucose. Incubation in the presence of glucose, shifted each protein to a higher molecular weight, suggesting that carbohydrate was bound to the proteins. Western blot analysis(Fig. 2B) revealed immunostaining-bands for all proteins except phosphorylase b by two weeks although the intensity of staining was different for each protein. This result suggested that crossline structures form at different rates in different proteins. We next studied effect of pH on the formation of crossline. As shown in Fig. 3, crossline increased in a time-dependent manner at pH 7.2 and 9.0. At pH 5.0, crossline rapidly increased, reached to a maximum at the second week and gradually decreased thereafter. The sensitivity to pH suggests that crossline structures may degrade or change conformation after incubation for long periods at acidic pH.

Crossline in rat lens proteins. Crossline was detected in preparation of rat lens proteins as measured by competitive ELISA (Fig. 4). The levels of crossline increased significantly with age and with the presence and duration of diabetes. At both 12 weeks and 20 weeks of age, the mean crossline content of lens protein preparations from diabetic rats was >2.3-fold higher than preparation from age-matched control rat.

Elevation and accumulation of AGEs *in vivo* play a central role in the pathogenesis of diabetic complications (1, 12-14). For determination of AGEs, fluorescence measurement at Ex 370/Em 440 nm and the immunological detection by antibodies to AGE-modified proteins have been used (15-17). However, the chemical structures of most natural fluorophores and of the epitope(s) recognized by anti-AGE antisera remain unclear. The fluorescence spectrum crossline (Ex 379/Em 463 nm) resemble that of the protein fluorophores existing *in vivo* (Ex 370/Em 440 nm). However, crossline is too acid-labile for direct isolation from hydrolyzed tissue. In the previous paper, we have shown immunohistochemically that crossline-like structure accumulates in renal tissues of rats with diabetic nephropathy (18). However, quantitative evaluation have been required in order to analys the importance of crossline *in vivo.* In this study, use of specific antibodies to the crossline haptens provide evidence that most AGE-modified proteins contain crossline-like structure(s). The *in vivo* formation of crossline increased with age and the concentration of crossline was increased in diabetes. Measurement of crossline-like structures in biological specimens may provide an index of aging and of the development of diabetic complications.

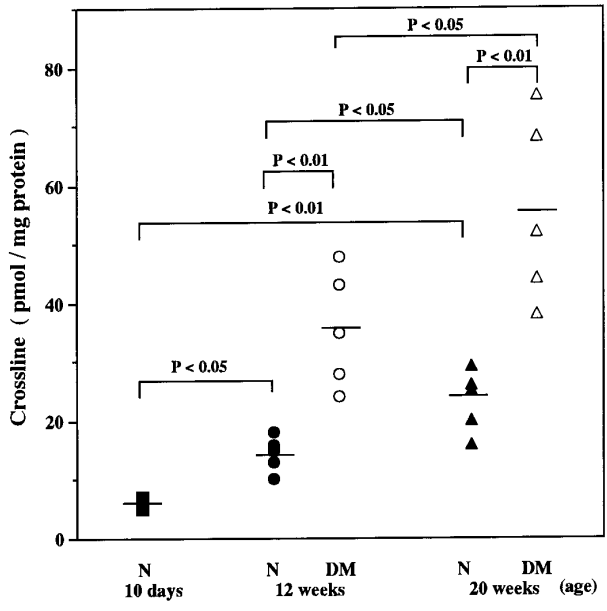

FIG. 4. Crossline in rat lens proteins measured by competitive ELISA. Nondiabetic control rats: ■, aged 10 days (n=2); ●, aged 12 weeks (n=5); ▲, aged 20 weeks (n=5). Diabetic rat (STZ injection at 8 weeks of age): ○, aged 12 weeks (n=5); △, aged 20 weeks (n=5). Horizontal bars indicate mean levels in each group. P values for comparisons were determined by unpaired Student's t-test.

ACKNOWLEDGMENT

This work was supported, in part, by a grant from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. Monnier, V. M., and Cerami, A. (1981) *Science* **211,** 491–494.
2. Brownlee, M., Vlassara, H., and Cerami, A. (1984) *Ann. Intern. Med.* **101,** 527–537.
3. Pongor, S., Ulrich, P. C., Bencsath, F. A., and Cerami, A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2684–2688.
4. Horiuchi, S., Shigeta, M., Araki, N., Tanaka, K., Saitoh, M., and Morino, Y. (1988) *J. Biol. Chem.* **263,** 18821–18826.
5. Njoroge, F. G., Fernandes, A. A., and Monnier, V. M. (1988) *J. Biol. Chem.* **263,** 10646–10652.
6. Shell, D. R., and Monnier, U. M. (1989) *J. Biol. Chem.* **264,** 21597–21602.
7. Nakamura, K., Hasegawa, T., Fukunaga, Y., and Ienaga, K. (1992) *J. Chem. Soc. Chem. Commun.* 992–994.
8. Hayase, F., Nagaraj, R. H., Miyata, S., Njoroge, F. G., and Monnier, V. M. (1989) *J. Biol. Chem.* **263,** 3758–3764.
9. Dunn, J. A., Patric, J. S., Thorpe, S. R., and Baynes, J. W. (1989) *Biochemistry* **28,** 9464–9468.
10. Laemmili, U. K. (1970) *Nature* **227,** 680–685.
11. Towbin, H., Staehlin, T., and Gordon, T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
12. Vlassara, H., Brownlee, M., and Cerami, A. (1986) *Clin. Chem.* **32/10**(B), B37–B41.
13. Brownlee, M. (1992) *Diabetes* **43,** 836–841.
14. Vlassara, H. (1992) *Diabetes* **41**(Suppl. 2), 52–56.
15. Nakayama, H., Taneda, S., Kuwajima, S., Aoki, S., Kuroda, Y., Misawa, K., and Nakagawa, S. (1989) *Biochem. Biophys. Res. Commun.* **162,** 740–745.
16. Horiuchi, S., Araki, N., and Morino, Y. (1991) *J. Biol. Chem.* **266,** 7329–7332.
17. Makita, Z., Vlassara, H., Cerami, A., and Bucala, R. (1992) *J. Biol. Chem.* **267,** 5133–5138.
18. Ienaga, K., Kakita, T., Hochi, T., Nakamura, K., Nakazawa, Y., Fukunaga, Y., Aoki, S., Hasegawa, G., Tsutsumi, Y., Kitagawa, Y., and Nakano, K. (1996) *Proc. Japan Acad.* **72B,** 79–84.