Advanced Glycation End Products of the Maillard Reaction in Aortic Pepsin-Insoluble and Pepsin-Soluble Collagen From Diabetic Rats

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Recent immunohistological studies using antibodies against advanced glycation end products (AGEs) have demonstrated the presence of AGEs in several tissues. By an enzyme-linked immunosorbent assay using the monoclonal anti-AGE antibody, the present study aimed to determine AGEs in pepsin-insoluble collagen (PIC) as well as in pepsin-soluble collagen (PSC) from the aortas of streptozotocin (STZ)-induced diabetic rats (at 4, 16, and 28 weeks after STZ injection) and those of age-matched control rats. Addition of EDTA to the immunoassay buffer has led us to successful determination of AGEs in the aortic PIC samples with following results: *1*) in diabetic rats, there was a time-related increase in the AGE contents at 28 weeks (n = 9, 226.4 ± 13.5 ng/mg collagen [mean ± SE]), compared with that at 4 and 16 weeks (n = 6, 79.6 ± 9.5 ng/mg collagen, and n = 8, 149.4 ± 30.9 ng/mg collagen at 4 and 16 weeks, respectively; both $P < 0.05$, between 4 and 16 weeks and 28 weeks); *2*) after 28 weeks of diabetes, the AGE contents in PIC of aortas were significantly higher in diabetic rats than in controls (n = 9, 226.4 ± 13.5 ng/mg collagen vs. n = 8, 129.6 ± 14.9 ng/mg collagen, $P < 0.01$, diabetic vs. control); and *3*) the level of the AGE content was strongly correlated with the PIC/total collagen (TC) ratio ($n = 45, r = 0.698, P = 0.0001$). By treating the samples of PSC with alkaline solution, the AGE content of PSC was also determined. In the PSC fraction, the AGE levels in the diabetic rats tended to increase with time and to be higher than those of control rats at 28 weeks although these changes were not statistically significant (diabetic: n = 4, 19.4 ± 9.7; n = 6, 22.3 ± 6.2; n = 6, 39.6 ± 10.8; control: n = 4, 19.7 ± 9.8; n = 6, 22.9 ± 7.3; n = 7, 30.7 ± 7.2; at 4, 16, and 28 weeks, respectively). Compared with the AGE levels of PSC, those of PIC were about four to seven times and four to five times higher in diabetic and control rats, respectively (PIC versus PSC in diabetic or control rats, all $P < 0.001$, at 4, 16, and 28 weeks, respectively). These findings provide the first immunochemical evidence that AGE adducts are present in the materials extracted sequentially by pepsin and collagenase and that these adducts in PIC accumulated as a function of the increase in the aortic PIC/TC ratio. *Diabetes* 45:1037–1043, 1996

Chronic hyperglycemia leads to the accumulation of proteins modified by glycation adducts. In this process, called the Maillard reaction, glucose first forms Amadori adducts with proteins (1,2), and some of these Amadori products, through a series of chemical rearrangements, are then converted to advanced glycation end products (AGEs), which continue to accumulate on long-lived proteins, such as lens crystallins (3), collagen (4,5), and myelin (6). It is generally accepted that a sustained hyperglycemia is a major factor in the development of diabetic complications such as micro- and macroangiopathy (1,7–9). A variety of experimental studies have also provided indirect evidence that AGEs could play a role in the structural and functional alterations in proteins during aging and long-term diabetes (5,7,8,10–12) and that AGE-mediated cross-link formation could contribute to a decrease in the solubility of collagen in the skin and tendons of humans or animals (4,13,14).

Recent immunochemical studies using anti-AGE antibodies have strongly suggested the presence of AGE adducts in vivo (15–18), although the in vivo presence of specific AGE structures such as 2-(2-furoyl)-4-(5)-(2-furanyl)-1H-imidazole (FFI) and pyrraline has been doubted in some reports (19–22). However, it is not known whether or not immunochemically reactive AGEs exist in tissue collagen when solubilized by extensive, multiple proteinase digestion. In the present study, we developed an enzyme-linked immunosorbent assay (ELISA) system with an AGE-specific monoclonal antibody to determine the amounts of AGEs in the aortic pepsin-insoluble collagen (PIC) extracted sequentially by two proteinases, pepsin and collagenase. The results of this study provide immunochemical evidence that the AGE adducts are present in the materials extracted by extensive proteinase digestion from the aortas of diabetic and age-matched control rats.

RESEARCH DESIGN AND METHODS

Reagents. Pepsin, bacterial collagenase (type VII), bovine serum albumin (BSA), native collagen (type IV and type I) and streptozotocin (STZ) were purchased from Sigma (St. Louis, MO). D-glucose was purchased

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AGE, advanced glycosylation end product; ANOVA, analysis of variance; BSA, bovine serum albumin; CML, N'-carboxymethyl-lysine; ELISA, enzyme-linked immunosorbent assay; FFI, 2-(2-furoyl)-4-(5)-(2-furanyl)-1H-imidazole; HRP, horseradish perioxidase; NBT, nitro blue tetrazolium; PBS, phosphate-buffered saline; PIC, pepsin-insoluble collagen; PSC, pepsin-soluble collagen; RSA, rabbit serum albumin; STZ, streptozotocin; TC, total collagen.

from Wako (Osaka, Japan). Horseradish perioxidase (HRP)-conjugated rabbit anti-mouse IgG antibody was purchased from Organon (West Chester, PA). The fructosamine assay kit was from Roche (Basel, Switzerland). All other chemicals and reagents used were of analytical reagent grade. Ninety-six–well microtitration plates (Nunc Immunoplate II) were purchased from Gibco (Grand Island, NY). AGE-BSA was prepared as described previously (23). AGE-collagen type IV was prepared in the same way as AGE-BSA. Briefly, collagen type IV (1.5 mg/ml) was incubated in 10 ml 0.5 mol/l phosphate-buffered saline (PBS) (pH 7.4) with 2 mol/l glucose at 37°C for 6 months under aseptic conditions, followed by dialysis against 20 mmol/l PBS (pH 7.4).

Animals. Forty-five Sprague-Dawley 4-week-old male rats, weighing 200–220 g, were randomly divided into two groups: STZ-induced diabetic rats and age-matched control rats. Diabetes was induced by an intravenous injection of 65 mg STZ per kg body wt, which was freshly prepared in 0.05 mol/l saline-citrate buffer (pH 4.5) before injection. The control rats were given the same volume of 0.05 mol/l saline-citrate buffer solution. Immediately after blood samples were obtained, the animals were killed under ether anesthesia at 4, 16, and 28 weeks after the start of the experiment. Before killing, 24-h urine samples were taken using a metabolic cage. The blood glucose was measured by the glucose oxidase–peroxidase method. All animal care and treatment conformed to our university's guidelines for the care and use of laboratory animals.

Tissue preparation. To prepare aortas from diabetic and age-matched control rats, the entire length of the aorta was cut into small pieces with a razor blade and rinsed twice with 4 mmol/l EDTA, 0.1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l *N*-ethylmaleimide, and 0.1 μg/ml pepstatin A. The pellets were then delipidated with a chloroform and methanol mixture (2:1, vol/vol) by gently shaking at 4°C for 24 h. After centrifugation at 5,000 rpm for 15 min, the pellets were washed sequentially, once with methanol and three times with deionized water. After drying with a freeze dryer (EYELA FD-80, Tokyo Rikakikai) for 24 h, the pellets were stored at −40°C until use.

Preparation and measurement of aortic pepsin-soluble collagen (PSC) and PIC. Ten-microgram samples were incubated with 1 mg/ml pepsin in 0.5 mol/l acetic acid by gently shaking at room temperature for 24 h. After centrifugation at 15,000 rpm at 4°C for 20 min, the clear supernatants were taken as the PSC fraction. The precipitate was then further incubated with gentle shaking with 270 U/ml of bacterial collagenase (type VII) in HEPES buffer (pH 7.4) containing 0.1 mol/l $CaCl_2$ for 24 h at 37°C, and 4 μl/ml of toluene:chloroform (1:1 vol/vol) was added to the collagenase solution to prevent bacterial growth. After centrifugation at 15,000 rpm at 4°C for 20 min, the supernatants were taken as the PIC fraction. The PSC and PIC samples were determined for AGE contents, fluorescence levels, and concentrations of fructosamine and hydroxyproline.

The determination of the collagen content was based on the assumption that hydroxyproline constitutes 14% of collagen. Hydroxyproline in PSC and PIC was measured according to Stegemann and Stalder (24). Briefly, a 50-μl sample was acid-hydrolyzed in 1 ml 6 N HCl at 100°C for 24 h. After the evaporation of hydrochloric acid, 4-hydroxyproline was measured in the dry residue with a spectrophotometer (Shimazu, Tokyo, Japan) at a wavelength of 550 nm. The ratio of PIC to the total content of the extracted collagen (TC) (which means the same as pepsin insolubility) from the aortas was calculated as follows: PIC/TC ratio = PIC content/total amount of solubilized collagen (PSC + PIC).

Preparation of monoclonal anti-AGE antibody. The monoclonal antibody against AGEs (anti-AGE antibody) was prepared by immunizing BALB/c mice with AGE-BSA as described previously (22). The antibody could recognize a structure(s) in common among AGE preparations obtained from proteins, polylysine, lysine derivatives, and monoaminocarboxylic acids, but did not recognize AGE structures proposed so far, which included FFI, pyrrole aldehyde, and pentosidine (22). In spite of intensive studies in several laboratories, the precise structure of the epitope recognized by the antibody remains unknown.

Determination of AGEs in the PIC and PSC by ELISA. A noncompetitive ELISA was used to examine the immunoreactivity of the monoclonal anti-AGE antibody with different AGE proteins such as AGE-BSA, AGE-collagen, and AGE–rabbit serum albumin (RSA). AGE contents were determined by a competitive ELISA. All assays were performed at room temperature. For the noncompetitive ELISA, each well of a 96-well microtiter plate was coated for 1 h with 100 μl 10 μg/ml of various AGE proteins, AGE-BSA, AGE-collagen, and AGE-RSA or their counterparts, in 50 mmol/l carbonate buffer (pH 9.6) for 1 h and washed three times with PBS containing 0.05% Tween 20 (buffer A, pH 7.4). Each well was then blocked with 150 μl 0.5% gelatin in 50 mmol/l carbonate buffer for 1 h, washed three times with buffer A, and then reacted with 100 μl of various concentrations of the primary anti-AGE antibody for 1 h. The wells were then washed three times with buffer A and incubated with 100 μl 1 μg/ml secondary HRP-conjugated rabbit anti-mouse IgG antibody for 30 min. After washing with buffer A, the wells were reacted with 1,2-phenylenediamine dihydrochloride as a colorimetric substrate. The reaction was terminated by the addition of 100 μl 1 mol/l sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader (Biometallics, Princeton, NJ).

The amount of AGEs in the PIC was determined by a competitive ELISA. The standard curve was constructed with AGE-BSA antigen. Each well of a 96-well microtiter plate was incubated overnight at 4°C with 50 μl 1 μg/ml AGE-BSA in 50 mmol/l carbonate buffer (pH 9.6). Each well was washed three times with buffer A and then blocked for 1 h with 150 μl 0.5% gelatin in 50 mmol/l carbonate buffer. After washing three times with buffer A, 50 μl AGE-BSA solution and AGE-BSA solution containing 270 U/ml collagenase type VII and/or 40 mmol/l EDTA was added to the wells as an inhibitor. Then 50 μl buffer A containing 0.1% BSA was added to two wells as a blank. Each well was incubated for 1 h with 50 μl 0.1 μg/ml of the primary antibody. The competitive ELISA procedure after the reaction with the primary antibody was performed in the same way as described for the noncompetitive ELISA. The samples to be assayed were pretreated with 40 mmol/l EDTA before the competitive ELISA. The AGE contents of the samples were determined by fitting the absorbance of the samples with the standard curve obtained from a competitive ELISA of AGE-BSA containing both 40 mmol/l EDTA and 270 U/ml collagenase.

The competitive ELISA to determine the amount of AGEs in the PSC fraction was performed in a fashion identical to that described above except that the samples were treated with 0.2 N NaOH (1:1, vol/vol) before the competitive ELISA to increase the AGE exposure as described previously (25) and that the standard curve was constructed with AGE-BSA in the presence of both 1 mg/ml pepsin, 0.5 mol/l acetic acid solution and 0.2 N NaOH (1:1, vol/vol). The data were expressed as nanograms AGE-BSA equivalents per milligram collagen. The intra-assay and interassay coefficients of variation of PSC were 2.6–6.2 and 5.5–7.9%, respectively, while those of PIC were 3.1–7.5 and 8.7–9.4%, respectively.

Measurement of fluorescence. Relative fluorescence intensities were analyzed in the same manner as previously described (25). Fluorescence was measured at a wavelength of 370/440 nm (excitation/emission) with a Shimazu fluorescence spectrofluorophotometer. A native collagen type I digested sequentially by pepsin and collagenase, as described above, was used as a reference, and the fluorescence intensities were defined as 1 U of fluorescence for PSC and PIC, respectively. The values of the fluorescence in PSC and PIC were expressed in arbitrary units per milligram collagen compared with their native collagen preparations.

Measurement of fructosamine. The determination of the fructosamine content was based on the characteristics of fructosamine that reduces nitro blue tetrazolium (NBT) in alkaline buffer (26). The fructosamine content was measured with an assay kit according the manufacturer's instructions (Roche). Briefly, 50 μl sample or standard solution was added to 1 ml carbonate buffer (0.1 mol/l, pH 10.8) containing 0.25 mmol/l NBT at 37°C; the absorbance at 550 nm was measured at 10 and 15 min after mixing and compared with that of standard of fructosamine derived from human serum. The concentration of fructosamine was calculated as: fructosamine (nanomoles per milliliter) = concentration of standard × ($\Delta E_s/\Delta E_c$), where ΔE_s and ΔE_c were the absorbance differences between 10 and 15 min of samples and standards, respectively. The values were expressed as nanomoles per milligram collagen.

Statistical analysis. All numerical data were expressed as means ± SE. The significance of the differences between two groups was analyzed by a unpaired Student's *t* test. The time-related changes in each group were analyzed with a one-way analysis of variance (ANOVA) and Scheffé's multiple comparison test. $P < 0.05$ was considered to be statistically significant. Linear regression analysis was also performed, and the correlation coefficients were determined.

RESULTS

Table 1 shows the characteristics of the animals, aortic fructosamine contents, and levels of relative fluorescence examined in this study. The diabetic rats had a significantly lower body weight, higher blood glucose, and urine volumes

TABLE 1
Characteristics of animals, aortic fructosamine contents, and levels of relative fluorescence intensities in the PIC and PSC fractions examined in the present study

	4 weeks		16 weeks		28 weeks	
	Control	Diabetes	Control	Diabetes	Control	Diabetes
n	6	6	8	8	8	9
Body weight (g)	387.0 ± 14.0	249.8 ± 14.1†	647.5 ± 30.7	340.5 ± 24.5†	654.6 ± 19.4	291.8 ± 18.9†
Blood glucose (mg/dl)	203.3 ± 17.4	765.5 ± 63.6†	247.1 ± 15.0	766.2 ± 22.2†	176.0 ± 9.1	717.5 ± 13.4†
Urine volume (ml/24 h)	22.6 ± 2.4	96.1 ± 24.9*	15.9 ± 2.8	161.2 ± 28.6†	7.7 ± 0.8	114.1 ± 10.2†
Fluorescence (AU/mg)						
PIC	3.7 ± 0.6	3.8 ± 0.4	4.8 ± 0.3	5.4 ± 0.6	4.3 ± 0.2	5.7 ± 0.8
PSC	3.4 ± 0.3	3.3 ± 0.2	3.7 ± 0.3	4.5 ± 0.5	4.3 ± 0.3	5.4 ± 0.4*
Fructosamine (nmol/mg)						
PIC	40.9 ± 4.5	68.1 ± 4.6	41.5 ± 7.8	74.9 ± 13.0*	54.7 ± 8.3	74.1 ± 7.7
PSC	38.9 ± 4.7	46.0 ± 7.1	37.9 ± 3.2	70.4 ± 7.0†	34.6 ± 2.6	60.5 ± 6.0†

Data are means ± SE. *$P < 0.05$, significantly different from the age-matched controls by Student's unpaired *t* test. †$P < 0.01$, significantly different from the age-matched controls by Student's unpaired *t* test.

than the age-matched control rats at 4, 16, and 28 weeks. The aortic fructosamine concentration of PSC as well as PIC was significantly higher in diabetic than in the control rats. It seems that the tissue fructosamine in the diabetic rats reached an equilibrium at 16 weeks and did not increase thereafter. The relative fluorescence level of PSC in the diabetic rats was higher than that in control rats at 28 weeks and the fluorescence levels of PIC in diabetic rats also tended to be higher than those in the control rats (Table 1).

The immunoreactivity of the monoclonal anti-AGE antibody with different AGE proteins such as AGE-BSA, AGE-collagen type IV, and AGE-RSA was initially examined in a noncompetitive ELISA. As shown in Fig. 1, the serial dilution curves of AGE-BSA, AGE-collagen type IV, and AGE-RSA almost overlapped each other, whereas the unmodified BSA and collagen did not react with the antibody in this system, indicating that a common AGE epitope(s) of these proteins was recognized by the monoclonal antibody.

To determine the AGE content in the PIC fraction by the competitive ELISA, we constructed a working standard curve with AGE-BSA and examined the effect of collagenase and EDTA on this dilution curve. As shown in Fig. 2*A*, the immunoreaction of AGE-BSA (AGE-BSA alone) with antibody was significantly interfered with by collagenase (AGE-BSA with collagenase). However, this interference was neutralized by the addition of 40 mmol/l EDTA in the reaction mixture (AGE-BSA with both collagenase and EDTA). The serial dilution curve of AGE-BSA in the presence of collagenase and 40 mmol/l EDTA was indistinguishable from the standard curve. Therefore, the AGE contents of the PIC samples containing 270 U/ml collagenase were determined by the competitive ELISA in the presence of 40 mmol/l EDTA with the serial dilution curve of AGE-BSA solution containing both 270 U/ml collagenase and 40 mmol/l EDTA as a standard one.

The nonspecific inhibition of pepsin/0.5 mol/l acetic acid solution to the antibody (AGE-BSA; BSA with pepsin/0.5 mol/l acetic acid) was observed in the ELISA system for the PSC fraction as shown in Fig. 2*B*. However, this nonspecific inhibition could be neutralized by alkaline solution (AGE-BSA; BSA with both pepsin/0.5 mol/l acetic acid and 0.2 N NaOH). The serial dilution curve of AGE-BSA in the presence of 1 mg/ml pepsin, 0.5 mol/l acetic acid and 0.2 N NaOH was almost indistinguishable from the standard curve (AGE-BSA alone; BSA). Therefore, the AGE contents of the PSC samples to be assayed were treated with 0.2 N NaOH (1:1, vol/vol) before the competitive ELISA.

Figure 3 shows the time course of the AGE contents of PIC and PSC in the diabetic and control rats. In the PIC fraction, *1*) the diabetic rats showed a significant increase in AGE contents at 28 weeks (n = 9, 226.4 ± 13.5 ng/mg collagen) compared with that at 4 and 16 weeks (n = 6, 79.6 ± 9.5 ng/mg collagen; n = 8, 149.4 ± 30.9 ng/mg collagen; at 4 and 16 weeks, respectively) (both $P < 0.05$, between 28 weeks

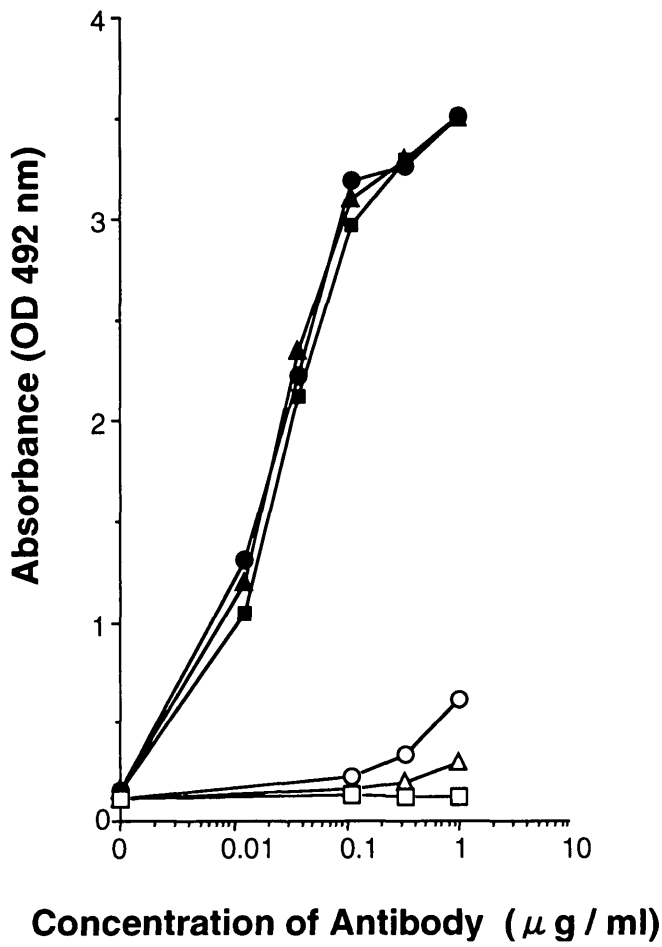

FIG. 1. Dilution curves for the monoclonal anti-AGE antibody. Monoclonal antibody was titered in a noncompetitive ELISA assay using AGE-BSA (●), AGE-collagen (■), AGE-RSA (▲), and their counterpart proteins BSA (○), collagen (□), and RSA (△) as absorbed antigens.

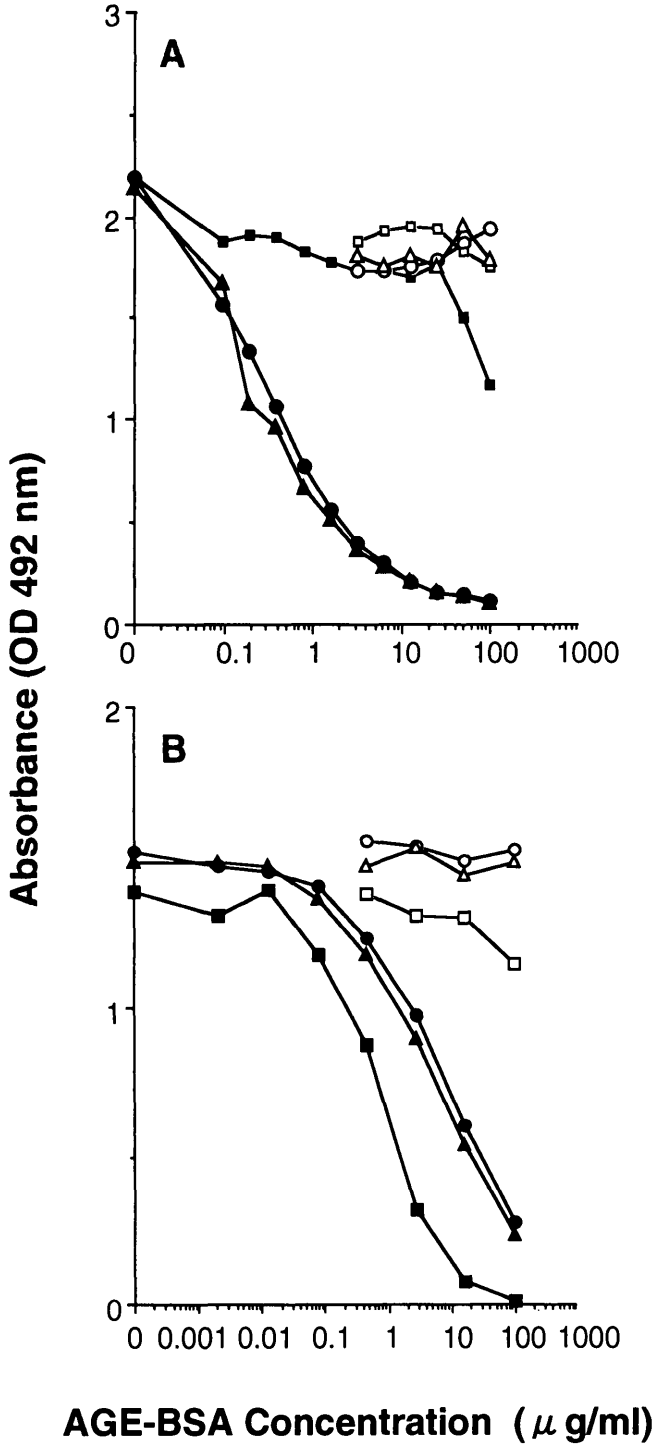

FIG. 2. *A:* effect of collagenase and EDTA on the immunoreactivity of the antibody to AGE-BSA. The immunoreaction of the antibody to various concentrations of AGE-BSA was performed in the absence (AGE-BSA alone, ●) or presence of collagenase (AGE-BSA with collagenase, □) and 40 mmol/l EDTA (AGE-BSA with both collagenase and 40 mmol/l EDTA, ▲). BSA, ○; BSA with collagenase, □; BSA with both collagenase and 40 mmol/l EDTA, △. *B:* nonspecific inhibition of pepsin, 0.5 mol/l acetic acid solution to the antibody (AGE-BSA, ■; BSA with pepsin/0.5 mol/l acetic acid, □) and the effect of 0.2 N NaOH on eliminating the nonspecific inhibition (AGE-BSA, △; BSA with both pepsin, 0.5 mol/l acetic acid and 0.2 N NaOH, ▲). AGE-BSA alone, ●; BSA, ○.

and 4 and 16 weeks); *2*) the AGE contents tended to increase with time in control rats although the change was not statistically significant, with values of 96.1 ± 15.8 (n = 6), 100.7 ± 22.5 (n = 8), and 129.6 ± 14.9 (n = 8) ng/mg collagen at 4, 16, and 28 weeks, respectively; and *3*) the AGE contents in diabetic rats were significantly higher than that in the control rats at 28 weeks ($P < 0.01$). In the PSC fraction, only a nonsignificant increase in the AGE levels in both the diabetic and control rats was observed (diabetic: n = 4, 19.4 ± 9.7; n = 6, 22.3 ± 6.2; n = 6, 39.6 ± 10.8; control: n = 4, 19.7 ± 9.8; n = 6, 22.9 ± 7.3; n = 7, 30.7 ± 7.2; at 4, 16, and 28 weeks, respectively). Compared with the AGE levels of PSC, those of PIC were about four to seven times and four to five times higher in the diabetic and control rats, respectively (PIC versus PSC in diabetic or control rats, all $P < 0.001$ at 4, 16, and 28 weeks, respectively).

The relation of the AGE adducts to PIC/TC ratio was also studied to characterize the common AGE epitope(s) present in the aortic collagen extracted by pepsin and collagenase. The total content of collagen (PSC plus PIC) extracted from the aorta showed neither any time-related changes nor differences between the diabetic and control rats (Fig. 4*A*). The values of the TC per milligram of tissue in control rats were 0.530 ± 0.044 (n = 6), 0.573 ± 0.022 (n = 8), and 0.586 ± 0.014 (n = 8) mg/mg total weight at 4, 16, and 28 weeks, respectively; and those in diabetic rats were 0.553 ± 0.028 (n = 6), 0.554 ± 0.034 (n = 8), and 0.586 ± 0.010 (n = 9) mg/mg tissue weight at 4, 16, and 28 weeks, respectively.

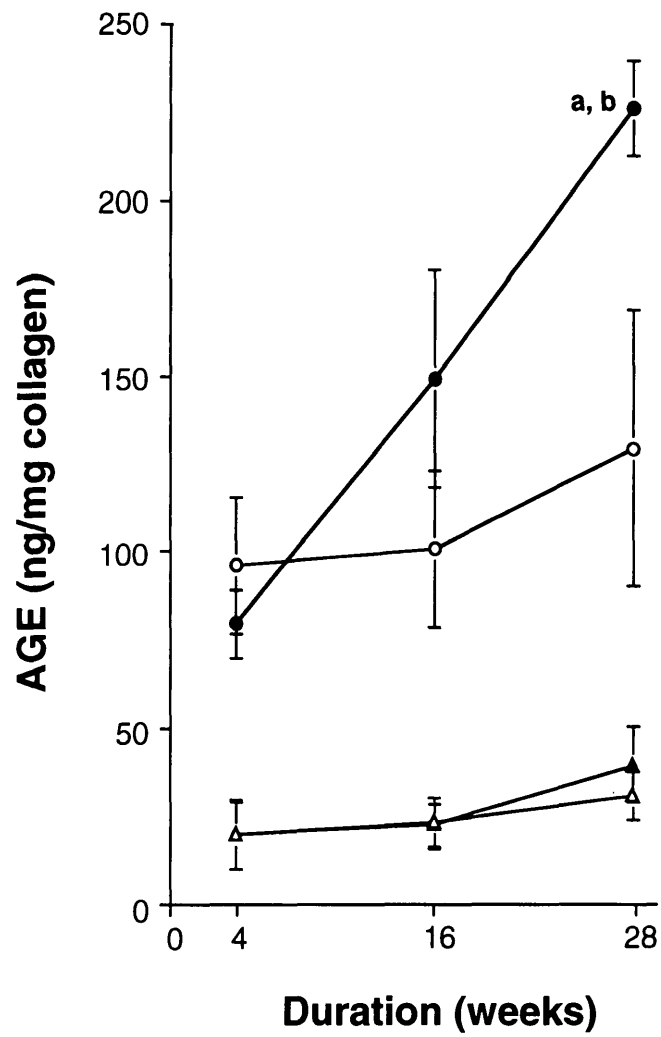

FIG. 3. The time-course of the AGE contents of PIC (control, ○; diabetic, ●) and PSC (control, △; diabetic, ▲) determined by a competitive ELISA assay. [a]$P < 0.01$, diabetic rats versus age-matched control rats by an unpaired Student's t test. [b]$P < 0.05$, 28 weeks versus 4 weeks and 16 weeks in the diabetic rats by ANOVA.

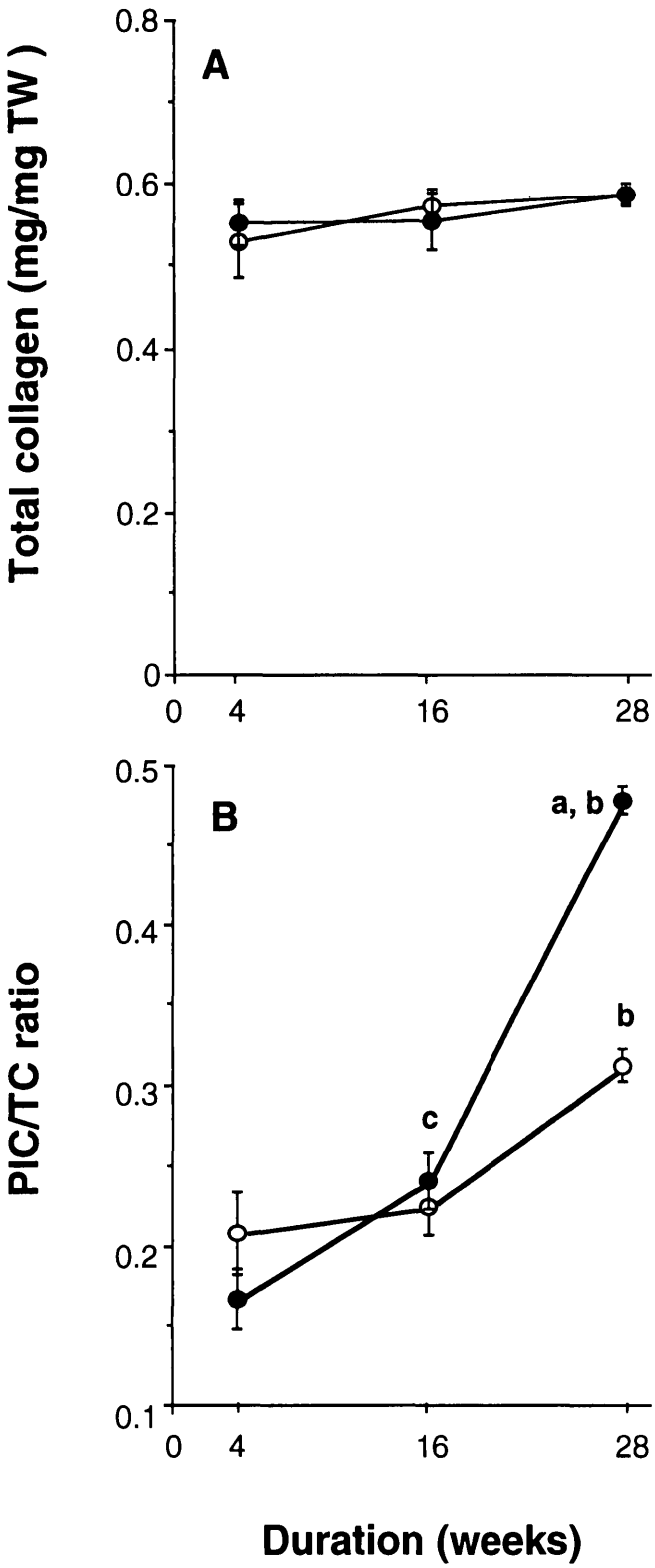

FIG. 4. TC (*A*) and the PIC/TC ratio (*B*) in the diabetic (●) and control rats (○). TC was the sum of PSC and PIC. [a]*P* = 0.0001, significantly different from age-matched control rats by an unpaired Student's *t* test. [b]*P* < 0.01, significant difference between 4 or 16 weeks and 28 weeks by ANOVA. [c]*P* < 0.05, significant difference between 4 and 16 weeks in the diabetic rats by ANOVA.

Figure 4*B* shows a time-related increase in the PIC/TC ratio in both control and diabetic rats. In control rats, the PIC/TC ratio was significantly higher at 28 weeks (n = 8, 0.312 ± 0.01) than those at 4 weeks (n = 6, 0.208 ± 0.026) and 16 weeks (n = 11, 0.225 ± 0.018) ($P < 0.01$). In the diabetic rats, there was a significant difference in the PIC/TC ratio between 4, 16, and 28 weeks (n = 6, 0.167 ± 0.019; n = 8, 0.241 ± 0.018; n = 9, 0.478 ± 0.009; at 4, 16 and 28 weeks, respectively; $P < 0.01$ between 4 or 16 weeks and 28 weeks, and $P < 0.05$ between 4 and 16 weeks). The level of the PIC/TC ratio in the diabetic rats was significantly higher than that in the control rats at 28 weeks (P = 0.0001) (Fig. 4*B*).

The AGE contents of PIC correlated with the PIC/TC ratio (n = 45, r = 0.698, P = 0.0001) (Fig. 5*A*). The correlation was stronger in the diabetic rats alone (n = 23, r = 0.736, P = 0.0001) (Fig. 5*B*), and no correlation was observed between the PIC/TC ratio and the AGE contents of PIC in the control rats alone. No significant correlation was observed between the AGE contents and the level of fluorescence in either the PIC or PSC fraction.

DISCUSSION

One of the important findings in the present study is that a common structure(s) of the AGE adducts is present in materials extracted sequentially by pepsin and collagenase from the aortas of STZ-induced diabetic and age-matched control rats. Several AGE structures such as FFI, *N'*-carboxymethyl-lysine (CML), pyrraline, and pentosidine have been identified thus far by a variety of direct or indirect criteria. Since the antibody used in the present study was reported to be unreactive to FFI, pyrraline, and pentosidine (22), the common structure detected in PIC or PSC might differ from those compounds. Its identity as CML is not known at the present.

Because extensive proteolysis by pepsin and/or collagenase was involved in preparing the PIC and PSC fractions, it could be possible that the AGE adducts detected by the present study do not occur in vivo but rather reflect an artifact. However, this possibility seems to be unlikely. First, the monoclonal antibody is able to react almost equally with different AGE proteins such as AGE-BSA, AGE-RSA, and AGE-collagen, but not with unmodified BSA, RSA, or collagen (Fig. 1), thus suggesting that this antibody recognizes a common structure among AGE proteins. This immunochemical result in our study is consistent with those of the previous studies (22,25). The immunoreactive antigen in the PIC as well as PSC could also be recognized by this antibody, indicating that the same AGE structure was present in the aortic collagen. Secondly, the AGE content of PIC strongly correlated with the PIC/TC ratio. This characteristic is a well-known property of AGE collagen. In addition, the AGE levels of the PIC fraction determined by the ELISA assay were markedly higher than those of the PSC fraction. This result supports a relationship between AGE and collagen insolubility, i.e., AGEs, if they are cross-links, should be present at higher levels in PIC compared with PSC. Finally, the AGE adducts of PIC in diabetic rats increased with time and were significantly higher than those in the control rats (Fig. 3). It seems that this immunochemically reactive AGE is relatively stable to hydrolysis and proteinase digestion. Although the metabolic fate, turnover, and removal of AGE have not yet been completely elucidated, if the breakdown of AGE proteins occurs by means of hydrolysis and enzymatic digestion, loss of reactivity to these catabolic factors should lead to the abnormal accumulation of AGE in blood and tissue, which could thus greatly accelerate tissue damage.

We could determine the amount of AGE adducts in PIC by

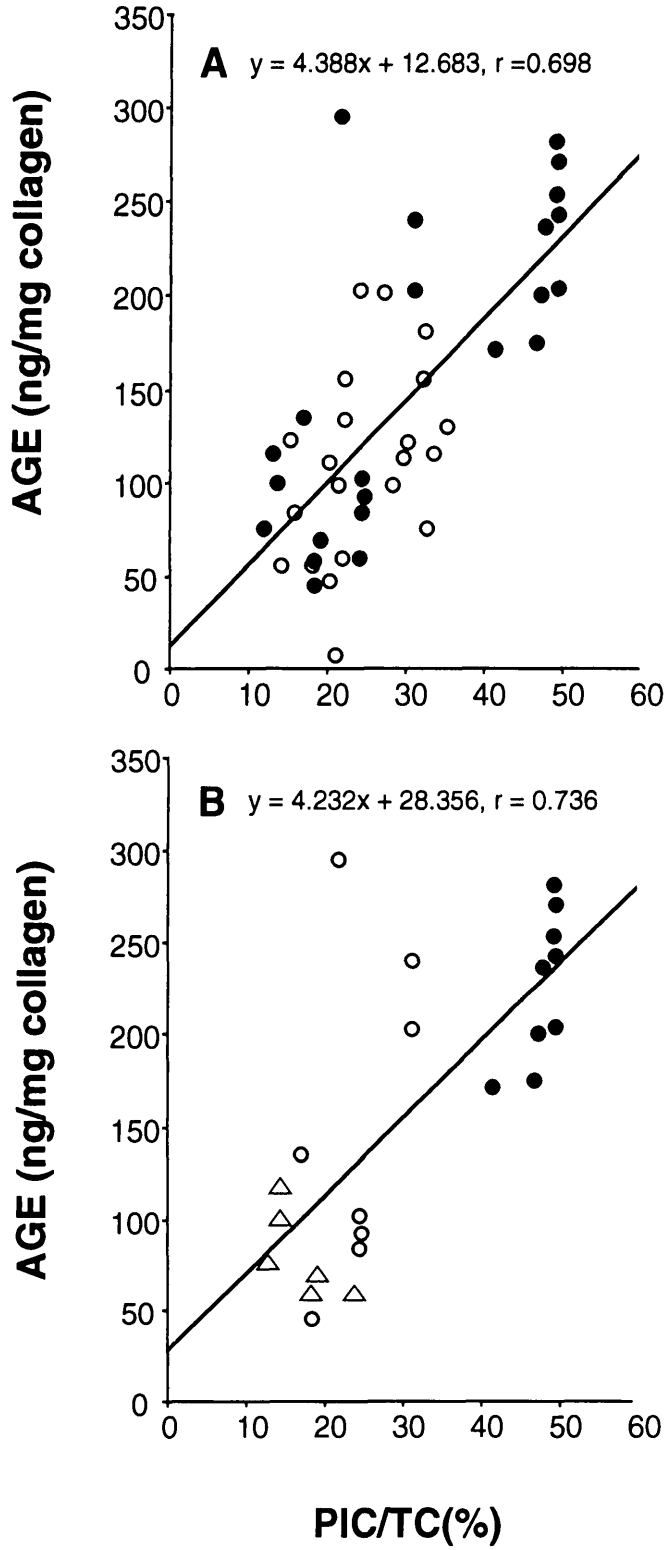

FIG. 5. *A:* **relation between the AGE contents of PIC determined by a competitive ELISA assay and the PIC/TC ratio in the diabetic (●) and control rats (○).** *B:* **correlation between the AGE contents of PIC determined by a competitive ELISA assay and the PIC/TC ratio in the diabetic rats alone with different durations of diabetes (at 4 [△], 16 [○], and 28 [●] weeks of diabetes).**

the addition of EDTA to the ELISA buffer in this study (Fig. 2*A*, AGE-BSA with both collagenase and EDTA, ▲). Without EDTA, the immunoreaction of the antibody with AGE-BSA was greatly hampered, probably by an insoluble precipitate formation between Ca^{2+} in collagenase buffer and phosphate in ELISA buffer (Fig. 2*A*, AGE-BSA with collagenase, ■). While the AGE in PSC was determined, a nonspecific inhibition was observed (Fig. 2*B*). Several attempts, including dialyzing the PSC samples against PBS and adding pepstatin A or alkali to the samples, were made to eliminate the influences derived from the preparation of PSC, such as pepsin or acetic acid to the ELISA assay. We found that 0.2 N NaOH was effective in eliminating the nonspecific inhibition, probably by neutralizing the acetic acid in the samples and increasing the AGE exposure as described previously (25).

In the present study, it is not known why the AGE contents as measured by the ELISA assay did not correlate with the level of the fluorescence. A similar result was also reported previously (25). One explanation for this finding is that several fluorescent compounds in body tissue can contribute to the level of fluorescence (27–29). Some fluorescent pigments originate from the oxidation and photo-oxidation of tissue (30,31). Since both glycation and oxidation contribute to protein modification in diabetes (32,33), the oxidative products could also yield tissue fluorescence. In addition, the fluorescence spectra obtained by irradiating human lens often overlap with the characteristic spectra of the products resulting from the incubation of BSA with glucose or ribose (30). For example, the emission wavelength at 440–460 nm of the human lens, which is presumably due to photo-oxidized products of tryptophan, coincides with the reported fluorescence peak of glycated BSA (29,30). Therefore, it is difficult to definitively characterize the AGE products in tissue by their fluorescence properties.

In summary, with the newly developed AGE-specific ELISA system, we obtained the first immunochemical evidence that AGE adducts are present in the materials extracted sequentially by pepsin and collagenase from the aortas of diabetic and age-matched control rats. These AGE adducts in PIC accumulated as a function of the increase in the aortic PIC/TC ratio.

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