Conversion of Amadori product of Maillard reaction to N^{ε}-(carboxymethyl)lysine in alkaline condition

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Abstract **N^{ε}-(carboxymethyl)lysine (CML) is known to be formed by oxidative cleavage of Amadori products between C-2 and C-3 of the carbohydrate chain. We report here that CML formation from Amadori compounds is highly accelerated under alkaline conditions. Incubation of glycated human serum albumin (HSA) in 0.1 N NaOH led to the formation of CML whereas glycated HSA reduced by $NaCNBH_3$ or non-glycated HSA did not generate CML. N^{α}-t-butyloxycarbonyl-N^{ε}-fructoselysine (Boc-FL), a model compound of Amadori product, was converted to CML under alkaline conditions. CML level of human sera ($n = 224$) preincubated with 0.1 N NaOH correlated well with glycated albumin value ($r = 0.912$) and hemoglobin A1c ($r = 0.797$).**

Key words: N^{ε}-(carboxymethyl)lysine (CML); Advanced glycation end product (AGE); Glycation; Glycoxidation; Amadori rearrangement product

1. Introduction

Long-term incubation of proteins with glucose leads, through the formation of early products such as a Schiff base and Amadori product, to advanced glycation end products (AGEs) which are characterized by fluorescence, brown color and cross-linking [1]. Immunological studies using a monoclonal anti-AGE antibody, 6D12 [2], not only demonstrated the presence of AGEs in vivo, but also suggested a potential link of AGEs to aging [3–5] and age-enhanced diseases such as diabetic complications [6,7], atherosclerosis [8–10], dialysis-related amyloidosis [11,12] and actinic elastosis of the photoaged skin [13]. Reddy et al. [14] identified N^{ε}-(carboxymethyl)lysine (CML) [15] as a major antigenic determinant in AGE proteins and our previous work also revealed that 6D12 recognized a CML protein adduct as an epitope [16], strongly suggesting an importance of CML among AGE structures in vivo. In addition to these immunological studies, the accumulation of CML-modified proteins in vivo has been demonstrated by chemical measurement [17–20].

CML is formed in three pathways in vitro, by oxidative cleavage of Amadori products [15,21] or Schiff bases [22,23] between C-2 and C-3 of the carbohydrate chain or by modification with glyoxal generated directly through autoxidation of glucose [24,25]. Above all, the first one is thought to represent a major pathway in vivo [26]. CML formation from Amadori product was accelerated by the addition of Fe^{3+} [27] and was inhibited by the presence of chelators, reducing agents, or radical scavengers [28–30]. We recently demonstrated that hydroxyl radical generated by the reaction of Fe^{2+} with H_2O_2 mediates CML formation from Amadori compounds [30]. CML is therefore termed 'glycoxidation product' among AGE structures and proposed as a potential biomarker of oxidative damage of tissue proteins in vivo [28,29].

Johnson et al. showed that 1-deoxy,1-morpholinofructose, a synthetic Amadori rearrangement product, increases its capacity to reduce nitroblue tetrazolium (NBT) with an increase in pH [31]. Furthermore, Sakurai et al. [32] later showed that the superoxide anion production from glycated polylysine (used as an Amadori product) in vitro occurs preferentially in alkaline medium. Based on these data, it seems reasonable to expect that CML formation from Amadori products is enhanced under alkaline condition. This possibility was tested in the present study.

2. Materials and methods

2.1. Subjects

Venous blood samples were obtained from 224 fasting healthy or diabetic subjects. Serum was separated by centrifugation at $1500 \times g$ at room temperature and stored at −80°C before determination. Anticoagulated whole blood was collected into EDTA-2Na-containing tubes to determine stable hemoglobin A1c (HbA1c).

2.2. Preparation of non-glycated and glycated standard

Non-glycated human serum albumin (HSA) was prepared by excluding glycated fraction from HSA [33]. One g of HSA (fraction V, Sigma, St. Louis, MO) was incubated with phenyl boronic acid resin (PBA-60, Amicon, Beverly, MA) in 800 ml of 0.5 M glycine-NaOH buffer containing 2% $MgCl_2$ (pH 8.5) for 2 h at room temperature. The filtrate through a glass filter was again incubated with another portion of the same resin. The finally obtained solution was concentrated to 30 mg/ml by an ultrafiltration system, followed by dialysis against phosphate-buffered saline (PBS) and was used for the experiments. The level of fructosamine in the non-glycated HSA (30 mg/ml) determined by fructosamine assay kit (Boehringer Mannheim, Mannheim, Germany) was negligible (10.9 μmol/l). To prepare glycated standard, a portion of the non-glycated standard solution was further concentrated to 80 mg/ml using an ultrafiltration system. We added 830 mg of D-glucose to the non-glycated standard solution (2.5 ml) followed by incubation with stirring at 56°C for up to 80 min. Time aliquots were taken and cooled on ice and dialyzed against PBS. The prepared solution (protein concentration of 30 mg/ml) was used as glycated standard in the present study. The fructosamine contents of normal HSA and glycated HSA (30 mg/ml) were 284 μmol/l and 1186 μmol/l, respectively.

2.3. Preparation of AGE-BSA

Bovine serum albumin (BSA, 50 mg/ml, fraction V, Sigma) was incubated with 2.0 M D-glucose in 10 ml of 0.5 M sodium phosphate buffer (pH 7.4) at 37°C for 1 month followed by dialysis against PBS.

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2.4. Incubation of glycated or non-glycated proteins in alkaline condition

Glycated or non-glycated standard HSA (2 mg/ml) was incubated in 0.1 N NaOH or PBS at 37°C for 16 h, followed by dialysis against PBS. In order to reduce Amadori products into hexitollysine, glycated proteins were preincubated at 4°C for 16 h in 1.0 ml of PBS containing 50 mM $NaCNBH_3$, followed by dialysis against PBS. Upon acid hydrolysis of glycated HSA and subsequent amino acid analysis identical to Fig. 2, we detected a peak corresponding to furosine with a retention time of 35.21 min. However, when glycated HSA was pretreated with $NaCNBH_3$, the peak of furosine disappeared but two peaks of hexitollysine (glucitollysine and mannitollysine) appeared between phenylalanine and lysine (data not shown), indicating that the Amadori product is reduced by $NaCNBH_3$. After the alkaline treatment, CML formation was determined by enzyme-linked immunosorbent assay (ELISA) and amino acid analysis. Human sera obtained from healthy or diabetic subjects were diluted at 1:80 with 0.1 N NaOH and were incubated at 37°C for 16 h.

2.5. Enzyme-linked immunosorbent assay (ELISA)

CML-HSA was prepared as described previously [14]. Briefly, 175 mg of HSA was incubated at 37°C for 24 h in 1.0 ml of 0.2 M sodium phosphate buffer (pH 7.8) containing 0.15 M glyoxylic acid and 0.45 M $NaCNBH_3$, followed by dialysis against PBS. A mouse monoclonal anti-AGE antibody, 6D12, which recognized CML protein adduct [2,16] was used to detect CML in the alkaline-treated samples. Assays were performed at room temperature by non-competitive and competitive ELISA [2,16]. In a non-competitive ELISA, each well of a 96-well microtiter plate (Immunoplate II, Nunc, Roskilde, Denmark) was coated with 100 μl of the sample to be tested in 50 mM sodium carbonate buffer (pH 9.6), blocked with 0.5% gelatin and washed three times with PBS containing 0.05% Tween 20 (washing buffer). Wells were incubated with 0.1 μg/ml of 6D12 (100 μl) dissolved in washing buffer for 1 h and then with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Kirkegaard Perry Laboratories, Gaithersburg, MD). When human sera were examined, biotin-labeled 6D12 and avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) were used to avoid cross-reaction between human IgG and anti-mouse IgG antibody. In a competitive ELISA, each well was coated with 100 μl of 10 μg/ml CML-HSA and was blocked with 0.5% gelatin. After washing three times with washing buffer, the wells were incubated with the sample to be tested (50 μl) and 0.1 μg/ml of 6D12 (50 μl) for 1 h. The wells were incubated with HRP-conjugated anti-mouse IgG antibody, followed by the reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1.0 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader. For experiments using ELISA, the same set of experiment was repeated at least three times and the representative data were shown in each figure.

2.6. Amino acid analysis

CML contents of modified proteins were quantitated by amino acid analysis as described previously [16,30]. After acid hydrolysis with 6 N HCl for 24 h at 110°C, samples were subjected to an amino acid analyzer (Model 835, Hitachi, Tokyo) using an ion-exchange HPLC column (#2622 SC, 4.6×60 mm, Hitachi Co.) and ninhydrine post-column detecting system. Hippuryl-CML was prepared by incubating hippuryllysine (benzoylglycyllysine, Peptide Institute, Osaka, Japan) with glyoxylic acid and $NaCNBH_3$ as described [16] and was used as a standard CML. The identity of CML detected by HPLC was confirmed by fast atom bombardment (FAB) mass spectrometry. The model Amadori compound, N^{α}-t-butyloxycarbonyl-N^{ε}-fructoselysine (Boc-FL) was synthesized as described previously [34]. The purity was assessed by a reverse phase HPLC (column: Shimadzu Techno Research STR-ODSII; eluent: 30% methanol in 0.1% TFA) and the structure was confirmed by ^{1}H-NMR (Bruker ARX-500) and sputtered ion mass spectrometry (Hitachi M-80B).

2.7. Determination of glycated albumin and stable HbA1c

The amount of glycated albumin was determined with glycated albumin assay kit (Labofit Glycoalbumin, Nacalai Tesque, Kyoto, Japan) [33]. To each well of a 96-well microtiter plate coated with anti-HSA antibody we added 50 μl of 10 mM phosphate buffer (pH 7.4) containing 0.05% Tween 20 and then 20 μl of the test sample. After incubation for 20 min, the wells were washed three times with 5 mM sodium carbonate buffer containing 2% $MgCl_2$ (washing buffer) and incubated for 20 min with the boronate-horseradish peroxidase conjugate in 50 μl of 100 mM glycine-NaOH (pH 9.0) containing 2% $MgCl_2$, 0.3% bovine hemoglobin and 0.05% Tween 20. The wells were then washed five times with the washing buffer and reacted for 20 min in the dark with 1 mg/ml o-phenylenediamine in 50 μl of 0.1 M citrate buffer (pH 5.8) containing 0.015% H_2O_2. The reaction was terminated by adding 50 μl of 2 N sulfuric acid. Absorbance (A) was measured at 492 nm by a microplate reader (MTP-120, Corona Electronic, Japan). Non-glycated standard albumin (NGA) and glycated standard albumin with a glycated albumin level of 40% (GA 40%) were used as standards to determine the level of glycated albumin in each sample. The percentage of glycated albumin in each sample (% GA) was calculated according to the formula:

$$\%\ \mathrm{GA} = \frac{A \text{ of sample} - A \text{ of NGA}}{A \text{ of GA } 40\% - A \text{ of NGA}} \times 40(\%).$$

Stable HbA1c of the anticoagulated whole blood was determined by an automated stable glycated hemoglobin analyzer (HLC-723GHbIII-S, Tosoh, Tokyo, Japan). The system is based on the high-performance ion-exchange liquid chromatographic method.

3. Results

3.1. Effect of alkaline treatment on the immunoreactivity of AGE-BSA

Using AGE-BSA, obtained by the incubation with glucose for 1 month, the effect of alkaline treatment on the immunoreactivity toward 6D12 was examined. After incubation in 0.1 N NaOH at 4°C for 16 h, the immunoreactivity of AGE-BSA was markedly increased compared with the incubation in PBS (Fig. 1A). Alkaline-treated BSA slightly but significantly reacted with 6D12, whereas PBS-treated BSA showed no immunoreaction. Because these samples did not react with non-immune mouse IgG used as a negative control for 6D12 (data not shown), the immunoreactivity with 6D12 was considered specific for CML. In a competitive ELISA (Fig. 1B), the immunoreactivity in alkaline-treated AGE-BSA was ∼100-fold higher than PBS-treated one. BSA showed significant immunoreaction only after the alkaline treatment. It was possible that CML moiety was exposed by the protein-degenerative effect of alkaline solution as suggested previously [35,36]. However, it might be due to additional formation of CML by alkaline treatment.

3.2. CML formation under alkaline condition determined by amino acid analysis

To test this, CML content of AGE-BSA was measured by amino acid analysis. CML content of alkaline-treated AGE-BSA (1.220 mol/mol of BSA) was significantly higher than PBS-treated one (0.085 mol/mol of BSA). It is likely, therefore, that additional formation of CML did occur under alkaline condition and CML might be formed by oxidative cleavage of protein-bound Amadori adducts [15,21]. To further confirm the formation of CML from Amadori compounds, the model Amadori compounds such as Boc-FL and glycated HSA were incubated under alkaline condition. When 1 nmol of Boc-FL was subjected to acid hydrolysis and amino acid analysis, 0.811 nmol of lysine residue was recovered as one of decomposition products, whereas CML was not detectable (<0.005 nmol) (Fig. 2A). However, after incubation in 200 μl of 0.1 N NaOH at 37°C for 16 h, 0.274 nmol of CML was formed (Fig. 2B). Since CML was not detectable in alkaline-treated glycated standard HSA, we prepared highly glycated HSA (high contents of Amadori products and low

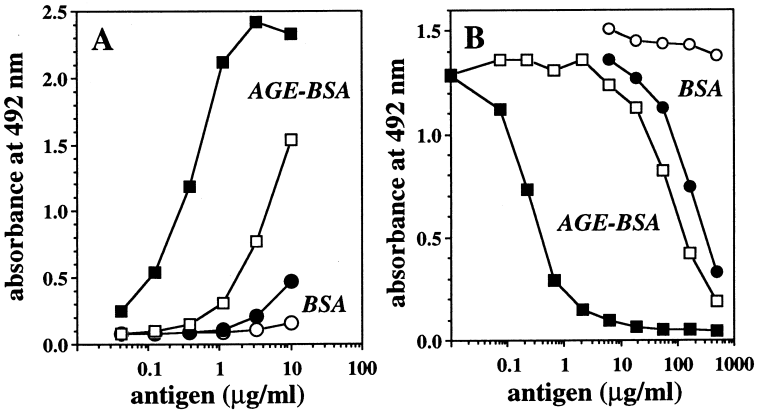

Fig. 1. Immunoreactivities of AGE-BSA and BSA incubated under alkaline or physiological condition were determined by non-competitive (A) and competitive (B) ELISA using 6D12. AGE-BSA (1.0 mg/ml) incubated in 1.0 ml of 0.1 N NaOH (■) or PBS (□) at 4°C for 16 h was subjected to ELISA. BSA was incubated in either 0.1 N NaOH (●) or PBS (○) in the same way.

level of CML) as described [29,30,37]. HSA (50 mg/ml) was incubated for 7 days at 37°C with 1.6 M glucose in 10 ml of 50 mM sodium phosphate buffer (pH 7.2) in the presence of 1 mM diethylenetriaminepentaacetic acid (DTPA). The fructosamine level of the highly glycated HSA (30 mg/ml) was 3189 μmol/l. CML content of this highly glycated HSA (2 mg/ml) was undetectable (Fig. 2C), whereas CML became a detectable level of 1.11 mol/mol of HSA after incubation in the same alkaline condition (Fig. 2D). CML was not generated by the alkaline treatment of non-glycated standard HSA for 16 h, nor by parallel incubation of highly glycated HSA with PBS (data not shown). These data indicated that CML formation from Amadori product in alkaline condition was faster than that in neutral conditions.

3.3. CML formation from Amadori products in glycated standard HSA and human sera under alkaline condition

This notion was further tested by determining the effect of alkaline condition on the CML formation from glycated HSA or human sera. As shown in Fig. 3A, alkaline treatment increased the immunoreactivity of glycated standards according to the glycated albumin levels, although PBS treatment had no effect on it. CML formation by the alkaline treatment was not observed in non-glycated standards (Fig. 3A), nor in glycated standard which had been preincubated with $NaCNBH_3$ in advance (data not shown). Similarly, alkaline treatment increased the immunoreactivity of human sera according to the glycated albumin levels (Fig. 3B). These data demonstrated that CML was artificially formed from protein-bound Amadori adducts under the alkaline condition. CML content of these alkaline-treated glycated standards or human sera was less than a detectable level of amino acid analysis (< 0.01 mol/mol of HSA).

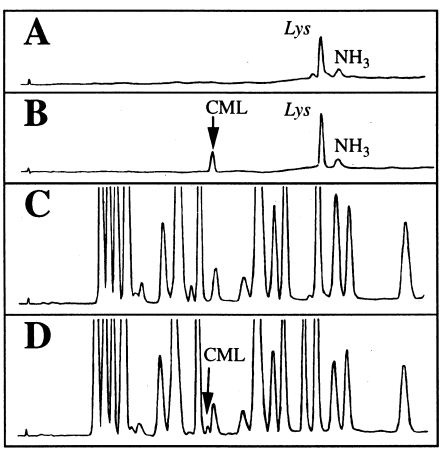

Fig. 2. CML formation under alkaline condition was determined by amino acid analysis. Boc-FL (1 nmol) was incubated in PBS (A) or 0.1 N NaOH (B) at 37°C for 16 h, followed by acid hydrolysis and amino acid analysis. Highly glycated HSA (2.0 mg/ml) was incubated in PBS (C) or 0.1 N NaOH (D) at 37°C for 16 h, followed by dialysis against PBS and was subjected to amino acid analysis. NH_3 represents a peak of ammonia.

3.4. The immunoreactivity of alkaline-treated human sera was specific for CML

To examine whether the immunoreactivity reflected the true amount of CML formed, the specificity was examined by competitive ELISA using CML-BSA or CML-modified peptide, hippuryl-CML, as standards. As shown in Fig. 4A, the immunoreaction of alkaline-treated human sera (B_0) was completely inhibited by CML-BSA and hippuryl-CML but not by their unmodified counterparts, BSA and hippuryllysine. On the other hand, the immunoreactivity of CML-BSA was significantly inhibited by the alkaline-treated human sera according to their glycated albumin levels (Fig. 4B). From these specific immunoreaction to CML, it is evident that the immunoreaction reflects the amount of CML formation in a specific manner.

3.5. Correlation between the immunoreactivity of the alkaline-treated human sera with 6D12 and glycated albumin level or stable HbA1c

Therefore, CML level of alkaline-treated human sera was determined by non-competitive ELISA using 6D12. It correlated well with the levels of glycated albumin (Fig. 5A). The regression line was $y = 0.0141x + 0.0849$ ($n = 224$, $r = 0.912$). This correlation again suggested that the immunoreactivity reflected CML formed from Amadori adducts of serum proteins under alkaline condition. CML formation was also correlated with HbA1c (Fig. 5B, $r = 0.797$).

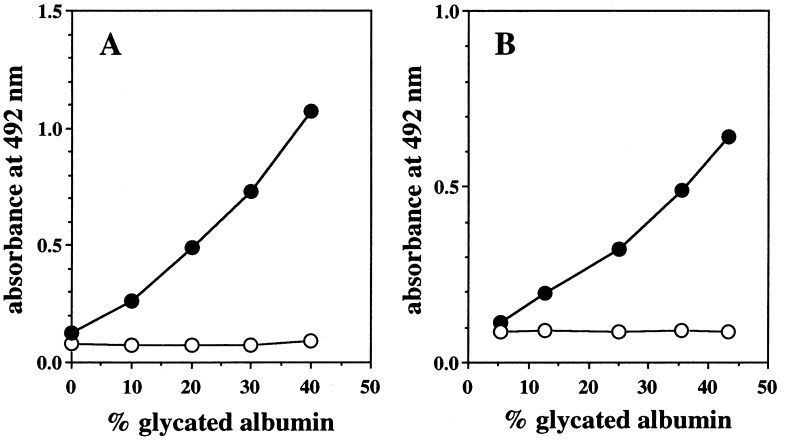

Fig. 3. CML formation in glycated standard HSA (A) or human sera (B) under alkaline condition was determined by non-competitive ELISA using 6D12. A: Glycated standard HSA with a glycated albumin level of 40% was mixed with non-glycated standard HSA to obtain the standards with glycated albumin level of 0, 10, 20 and 30%. These standards (1.0 mg/ml) were incubated in 1.0 ml of 0.1 N NaOH (●) or PBS (○) at 37°C for 16 h, followed by dialysis against PBS. B: Human sera obtained from healthy or diabetic subjects were diluted at 1:80 with 0.1 N NaOH (●) or PBS (○) and were incubated at 37°C for 16 h. CML formation in each sample at a fixed concentration (10 μg/ml) was determined by non-competitive ELISA. CML levels were expressed as the absorbance at 492 nm.

4. Discussion

We show here that under alkaline condition, CML is formed from Amadori compounds of in vitro glycated HSA and those of healthy or diabetic sera. Incubation of glycated HSA in 0.1 N NaOH led to the formation of CML whereas glycated HSA reduced by $NaCNBH_3$ or non-glycated HSA did not generate CML. CML level of human sera ($n = 224$) incubated with 0.1 N NaOH for 16 h at 37°C correlated well with glycated albumin value. Likewise, alkaline-treated BSA also significantly increased its reactivity to 6D12 (Fig. 1A and B). These data indicated that Amadori compounds of HSA or BSA were converted to CML under alkaline condition.

Tissue proteins showed strong immunoreactivity to anti-AGE antibodies after the treatment in alkaline condition [35,36]. In these studies, the immunoreactivity was obtained after the samples were incubated in 0.1 N NaOH solution for 12 h at 4°C and it was supposed that alkaline treatment might degenerate proteins so that AGEs exposed to the surface of the protein could easily be recognized by the antibody. However, as shown in the present study, it was possible that CML was artificially formed from Amadori products under alkaline condition.

The immunoreactivity of an alkaline-treated serum with 6D12 might reflect its amount of Amadori compounds, since it correlated very well with markers for glycemic control such as glycated albumin and HbA1c (Fig. 5). It was, therefore, likely that the contents of Amadori compound in human sera could be quantitated by ELISA using 6D12. It was also noted that the correlation coefficient was higher in glycated albumin than in HbA1c probably because the glycated albumin level

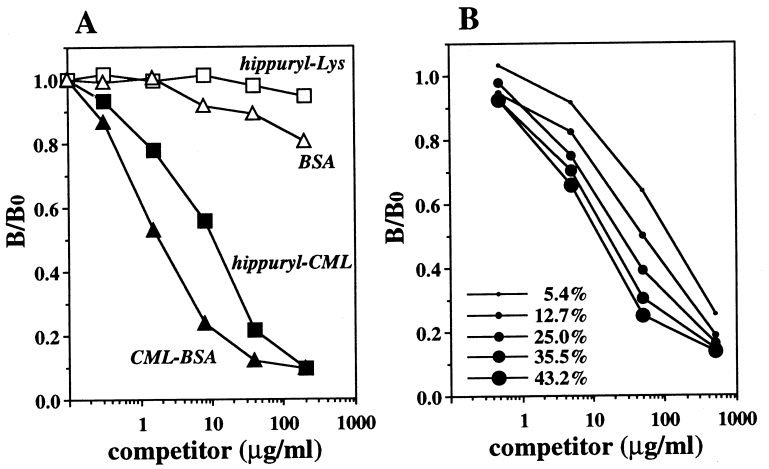

Fig. 4. Specificity of the immunoreaction of alkaline-treated human sera toward 6D12 was examined by competitive ELISA. A: Wells were coated with alkaline-treated diabetic serum with a glycated albumin level of 25.0% and were reacted with 6D12 in the presence or absence of competitors. Used competitors were: hippuryllysine (□), hippuryl-CML (■), BSA (△), and CML-BSA (▲). B: Wells were coated with 0.2 μg/ml CML-BSA and were reacted with 6D12 in the presence of alkaline-treated human sera. Human sera with the designated glycated albumin level of 5.4, 12.7, 25.0, 35.5 and 43.2% were used as competitors. The results were expressed as the ratio B/B_0 in which B represents the amount of binding of 6D12 in the presence of a competitor and B_0 represents that in the absence of it.

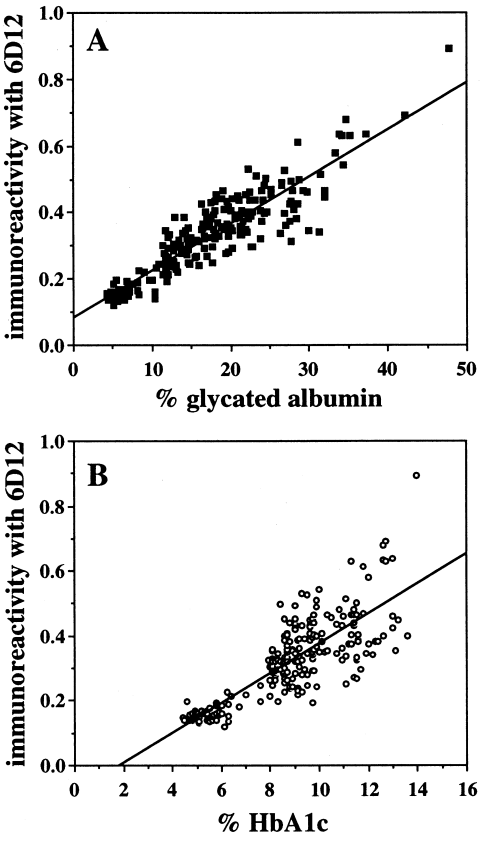

Fig. 5. Correlation between immunoreactivity of alkaline-treated human sera with 6D12 and glycated albumin (A) or stable HbA1c (B). Human sera were diluted at 1:80 with 0.1 N NaOH and were incubated at 37°C for 16 h. The immunoreactivity to 6D12 was examined by non-competitive ELISA and was expressed as the absorbance at 492 nm. A: $y=0.0141x+0.0849$ ($n=224$, $r=0.912$). B: $y=0.0458x-0.0804$ ($n=224$, $r=0.797$).

more directly reflected the amount of Amadori adducts which could be the source of CML in human sera.

Amadori products, also known as fructosamines, are formed by the Amadori rearrangement of Schiff base adducts of glucose with protein amino groups. Fructosamines are reductants under alkaline condition [31], a property used in the colorimetric assay of serum protein glycation, in which such proteins reduce the dye nitroblue tetrazolium [31]. Recent studies have demonstrated the generation of reactive oxygen species including O_2^- and H_2O_2 from Amadori products [21,32,38–40]. Furthermore, reactive oxygen species derived from Amadori compounds have been shown to be responsible for damage to amino acids, proteins, lipids, and DNA, which was catalyzed by transition metal ions [41–45]. Although the extreme alkaline conditions such as 0.1 N NaOH cannot be achieved in vivo even in some disease states, CML formation could be accelerated locally when glycated proteins are subjected to long-term exposure to mild alkaline conditions.

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