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Membrane Proteins of Human Erythrocytes Are Modified by Advanced Glycation End Products during Aging in the Circulation

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Recent immunological studies demonstrated that proteins *in vivo* in several diseases are subjected to post-translational modification by advanced glycation end products (AGEs), suggesting a potential role of AGEs in aging and age-enhanced disease processes such as diabetic complications, atherosclerosis and Alzheimer's disease. N^{ε}-(Carboxymethyl)lysine (CML) is one of the major AGE-structures demonstrated *in vivo* so far. In the present study, membrane proteins from young erythrocyte population were compared with those from senescent erythrocytes separated from the same individual in their CML-contents using a monoclonal antibody for CML (6D12). SDS-polyacrylamide gel electrophoresis and subsequent Western blot showed that 6D12 bound to the band 1, 2, 3, 4.2, 5, 6 and 7 proteins from senescent erythrocytes, but not to those from young erythrocytes. Furthermore, quantitative estimation of the reactivity of 6D12 to these erythrocyte membranes by ELISA showed that the reactivity of 6D12 to senescent erythrocyte membranes was 3- to 6-fold higher than that of young erythrocyte membranes. These results indicate that membrane proteins of circulating erythrocytes undergo CML-modification, and the modified proteins accumulated in an age-dependent manner during the life span of erythrocytes.

Long-term incubation of proteins with glucose leads, through the formation of early products such as a Schiff base and Amadori products, to advanced glycation end products (AGEs) which are characterized by fluorescence, brown color and intra- or inter-molecular cross-linking. Immunological studies using anti-AGE antibodies have shown that accumulation of AGEs increases with aging in human lens crystallins (1) and is also enhanced under several pathological conditions such as kidneys of patients with diabetic nephropathy (2) and chronic renal failure (3), atherosclerotic lesions of artery walls (4), amyloid fibrils in hemodialysis-related amyloidosis (5,6). These findings suggest the potential involvement of AGE modification in the pathogenesis of age-related disease processes. Several AGE structures have been identified including pyrraline (7), pentosidine (8), CML (9), CEL (10), imidazolone (11) and crosslines (12). Reddy *et al.* and our group revealed that CML is a major antigenic AGE structure (13,14). Furthermore, our recent studies demonstrated that CML levels of the sun-exposed skin area was significantly higher than that of the sun-unexposed area of the same individuals (15). Thus, AGE formation are preferentially proceeded in ultraviolet (UV)-exposed and photo-aged skin, implicating the active contribution of reactive oxygen species to the formation of AGEs.

Human erythrocytes differentiate from erythrocyte precursor cells in the bone marrow, circulate in the body for an average of 120 days, and are finally removed by spleen macrophages of the reticuloendothelial system. Plasma proteins such as albumin and proteins in erythrocytes such as hemoglobin undergo glycation. Membrane proteins in the erythrocytes may also be modified by glucose in the circulation during aging process. In the present study, we compared the levels of CML, one of major AGE-structures, of senescent erythrocyte membranes with those of young erythrocyte membranes by Western blotting and enzyme-linked immunosorbent assay (ELISA) using an antibody specific for CML.

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Abbreviations: AGEs, advanced glycation end products; BSA, bovine serum albumin; CBB, Coomassie brilliant blue R-250; CEL, N^{ε}-(carboxyethyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; DFP, diisopropyl fluorophosphate; DPBS, Dulbecco's phosphate-buffered saline; DPBS(-), Ca^{2+} and Mg^{2+}-free DPBS; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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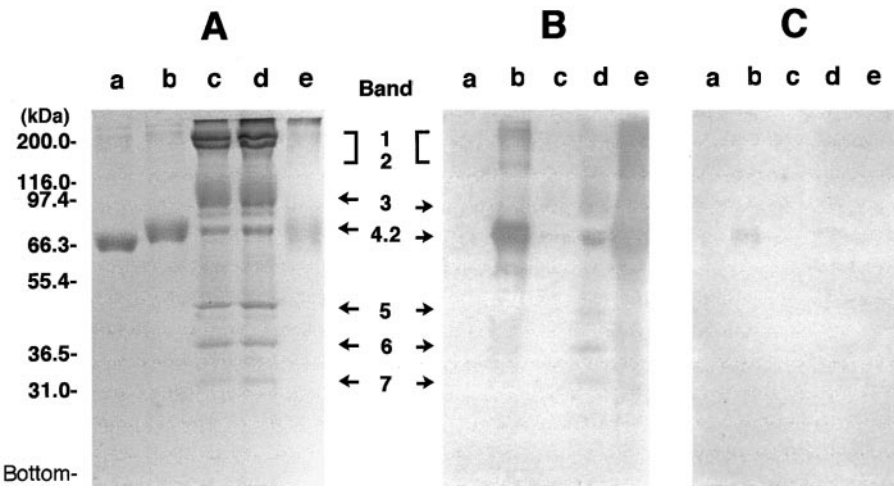

FIG. 1. Western blot analyses of membrane proteins of young and senescent erythrocyte. Young and senescent erythryocyte membrane proteins from donor 1 (blood group O) were used for the experiment. BSA (2.5 μg) (lane a), CML-BSA (2.5 μg) (lane b), AGE-BSA (2.5 μg) (lane e), young erythrocyte ghosts (40 μg) (lane c), and senescent erythrocyte ghosts (40 μg) (lane d) were subjected to SDS-PAGE under reducing condition. The gel was stained with CBB (panel A), or subjected to Western blotting using 6D12 to detect CML in the presence (panel B) or absence (panel C) of CML-BSA, followed by ^{125}I-goat anti-mouse IgG binding assay as described under Materials and Methods.

MATERIALS AND METHODS

Preparation of modified BSA. AGE-BSA was prepared as described previously (16). Briefly, 1.6 g of BSA and 3.0 g of glucose were dissolved in 10 ml of 0.5 M sodium phosphate buffer (pH 7.4), and the solution was sterilized by filtration (0.45 mm), and incubated at 37°C for 90 days. To prepare CML-BSA, 50 mg/ml of BSA was incubated at 37°C for 24 h with 45 mM glyoxylic acid and 150 mM $NaCNBH_3$ in 10 ml of 0.2 M sodium phosphate buffer (pH 7.4), followed by dialysis against DPBS (−) containing 0.05% NaN_3 (14). The extent of modification was assessed by determination of free amino-residues using the fluorescamine method (17). The extent of modification of amino residues in AGE-BSA and CML-BSA were 94.4% and 62.0%, respectively. The amount of protein was determined by the Lowry method (18).

Preparation of monoclonal anti-AGE antibody. The monoclonal anti-AGE antibody (6D12) was prepared as described previously (19). Briefly, splenic lymphocytes from Balb/c mouse immunized with AGE-BSA were fused to myeloma P3U1 cells. The hybrid cells which showed positive to AGE-BSA but negative to BSA was selected through successive subcloning. One line, termed 6D12, was produced from ascites of Balb/c mice and further purified by protein G affinity chromatography to IgG1. Our recent experiments showed that its epitope was CML-protein adducts (14).

Separation of young and senescent erythrocyte membranes. Erythrocytes were suspended in DPBS (−) at a concentration of 80%, and young and senescent erythrocytes were separated by density gradient centrifugation on Percoll gradient (20) as described previously (21). Young and senescent erythrocytes were washed several times with DPBS (−) by centrifugation at 320 × g for 10 min to remove Percoll and then washed. Cells were lyzed with 5 mM sodium phosphate buffer (pH 8.0) containing 20 μg/ml PMSF and washed several times with the same buffer by centrifugation at 9,600 × g for 20 min to obtain white ghosts, and resuspended in the same buffer containing DFP. The amount of protein was determined by the Lowry methods (18).

Identification of CML-modified erythrocyte membrane proteins by Western blotting. White ghosts were solubilized with 2% SDS for 30 min at 4°C and mixed with an equal volume of electrophoresis buffer of Laemmli, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (22) using 10% polyacrylamide gel under the reducing conditions. One gel was stained by Coomassie brilliant blue R-250 (CBB) and the other gel was subjected to Western blotting according to the method of Towbin *et al.* (23), and the protein bands on the SDS-PAGE gel were transferred to a PVDF membrane. The membrane was blocked with 0.3% BSA in DPBS (−) containing 0.02% NaN_3 at 4°C overnight, followed by washing twice with DPBS (−) and treatment with 1 ml of 6D12 (3 μg/ml) in 0.5% BSA in DPBS (−) containing 0.02% NaN_3 for 2 h at room temperature. In parallel experiments, the membranes were similarly treated with 1 ml of 6D12 (3 μg/ml) containing 11.2 mg/ml of CML-BSA. These membranes were rinsed four times with DPBS (−) containing 0.02% NaN_3 for 5 min, and then gently swirled in 10 ml of ^{125}I-labeled goat anti-mouse IgG (0.04 μg/ml) or ^{125}I-labeled goat anti-rabbit IgG (0.004 μg/ml) in 1% BSA in DPBS (−) containing 0.02% NaN_3 for 2 h at room temperature. The membranes were rinsed six times with 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 0.01% Triton X-100 for 15 min. The membranes were air-dried, and the radioactivity bound to these membranes was measured by autoradiography using a Fujix BAS 2000 Bio-Imaging Analyzer (Tokyo, Japan).

ELISA procedure. Young and senescent erythrocyte ghosts were delipidated by acetone and solubilized with 1% SDS, and the amount of solubilized proteins was determined by bicinchoninic acid method (24). The SDS-solubilized ghost solution was diluted in 50 mM sodium carbonate buffer (pH 9.5) to make various concentrations of samples. ELISA assays were performed at room temperature in a non-competitive ELISA. In a non-competitive assay, each well was incubated for 1 h with 0.1 ml of samples to be tested or its corresponding control sample in 50 mM carbonate buffer (pH 9.5) and washed three times with PBS containing 0.05% Tween 20 (buffer A). Each well was then blocked for 1 h with 0.2 ml of 0.5% gelatin in 50 mM carbonate buffer (pH 9.5). Each well was washed three times with buffer A and incubated for 1 h with 0.1 ml of 6D12 (1 μg/ml). Wells were then washed three times with buffer A and incubated for 1 h with 0.1 ml of horseradish peroxidase (HRP) labeled anti-mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by 1 M sulfuric acid and the absorbance at 492 nm was read on a micro-ELISA plate reader (Titertek Multiscan PLUS MKII).

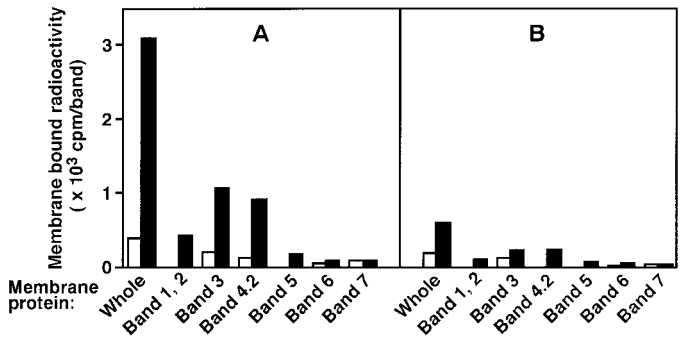

FIG. 2. Binding of ^{125}I-labeled 6D12 to membrane proteins blotted to PVDF membranes. The extent of the binding of 6D12 to blotted membrane proteins from young (open bars) and senescent (closed bars) erythrocyte in the presence (A) or absence (B) of CML-BSA were determined by counting each band using a Bio-Imaging Analyzer.

RESULTS

Erythrocytes of three healthy donors were separated into young and senescent erythrocytes by Percoll density gradient centrifugation, and the young and senescent ghosts were subjected to SDS-polyacrylamide gel electrophoresis, followed by CBB staining. Representative data obtained from donor 1 (blood group O) are shown in Fig. 1A. To detect whether these erythrocyte membranes underwent CML-modification, Western blotting was carried out using 6D12 and ^{125}I-goat anti-mouse IgG (Fig. 1B). 6D12 did not react with membrane proteins of young erythrocytes (lane C). However, it reacted strongly with bands 3, 4.2, and weakly to bands 1, 2, 5, 6, and 7 of senescent erythrocyte membranes (lane D). Because the major epitope of 6D12 is CML (14), competitive inhibition of the antibody with CML-BSA was investigated. The amounts of 6D12 bound to these membranes were significantly reduced by CML-BSA (Figs. 1C and 2B). Similar results were obtained from erythrocytes from other donors (blood group A and B) (data not shown). Membrane proteins of young and senescent erythrocytes from three different donors were compared for their immunoreactivity toward 6D12 by ELISA. The reactivity of senescent cell membranes to this antibody was about 3- to 6-fold higher than that of young cell membranes (Fig. 3). The results were consistent with those obtained by Western blotting (Fig. 1), strongly suggesting that modification with CML does occur to senescent erythrocyte membranes.

These data support the notion that modification by AGE-structures such as CML does occur to membrane proteins of human erythrocytes during aging in the circulation.

DISCUSSION

The present study demonstrated that membrane proteins from senescent erythrocytes underwent modification with CML, one of major AGE-structures. Furthermore, extents of CML-modification of these membrane proteins were significantly higher 3 to 6-fold than those from young erythrocytes obtained from the same individual. This indicates these membrane proteins undergo AGE-modification by CML time-dependently during aging of erythrocytes after entering the circulation from bone marrow. Thus, it is possible that CML is a potential marker of aging of erythrocytes in the circulation.

CML is formed in three pathways *in vitro* in the Maillard reaction. As shown in Fig. 4, the first is oxidative cleavage of Amadori products between C-2 and C-3 of the carbohydrate chain in the presence of metal ions (9), the second is oxidation of Schiff base to yield an alkylimine, which is the Schiff base adduct of glycolaldehyde with amine (25,26), and the third pathway is reaction between protein and glyoxal generated directly through autoxidation of glucose (27). Amadori adducts may be more likely precursor for CML *in vivo* (28). Oxidative mechanisms are suggested to be associated with the CML formation because this formation is suppressed by antioxidants (29), and hydroxyl radical can mediate CML formation from Amadori adducts (30). Other studies have demonstrated that CML is also formed during metal-catalyzed oxidation of polyunsaturated fatty acids in the presence of proteins, and suggested that lipid oxidation may produce CML in tissue proteins *in vivo* (31). Hence, CML detected in senescent erythrocyte membranes may be derived from two pathways: glycation of membrane proteins and

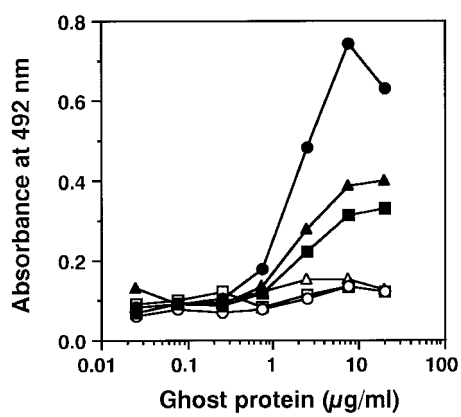

FIG. 3. Immunoreactivity of 6D12 with the membrane proteins of young and senescent erythrocytes by ELISA. Solutions of solubilized ghost protein at the indicated concentrations (0.1 ml) were incubated in 50 mM sodium carbonate buffer (pH 9.5) for 60 min. in a 96-well microtiter plate. Each well was washed and blocked with 0.5% gelatin. After washing, 0.1 ml of 6D12 (1 μg/ml) was reacted for 60 min. The wells were then washed and reacted with HRP-conjugated anti-mouse IgG followed by reaction with 1,2-phenylenediamine dihydrochloride. The test samples were young (○) and senescent (●) erythrocyte membranes from donor 1 (blood group O), young (△) and senescent (▲) erythrocyte membranes from donor 2 (blood group A), and young (□) and senescent (■) erythrocyte membranes from donor 3 (blood group B).

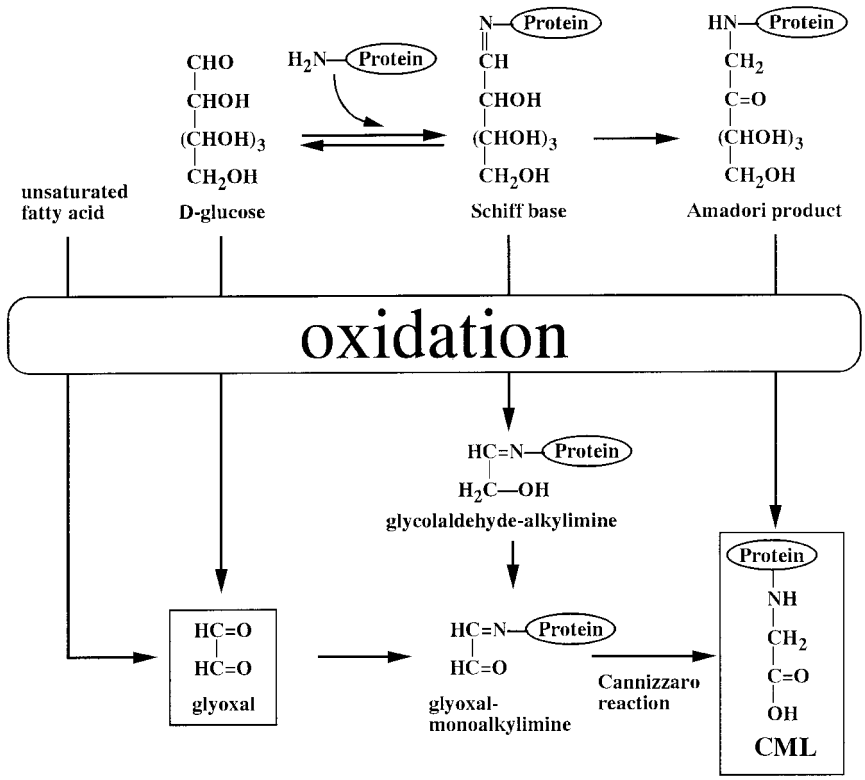

FIG. 4. Possible pathway for CML formation.

subsequent metal-dependent oxidation, lipid oxidation of membranes and subsequent reaction of the oxidation products with proteins, or both.

It has been demonstrated that AGE-proteins are recognized by the scavenger receptors of macrophages (16). AGEs in senescent erythrocyte membranes can function as target for macrophage scavenger receptors, which may eventually result in the phagocytic removal of scenescent erythrocytes from the circulation (32,33). There is another possibility that AGEs are involved in the mechanisms of removal of scenescent erythrocytes from the circulation. Human erythrocyte membranes exposed to oxidative stress in the circulation are known to undergo various oxidative modifications during aging in the circulation, which include polymerization of membrane proteins (34-37), aggregation of membrane proteins (38,39), production of fluorescent phospholipids (34,35,40), carbonyl groups (41), and lipid hydroperoxide and thiobarbituric acid-reactive substances (42). Upon oxidative damage, glycoproteins including band 3 were clustered to induce assembly of poly-N-acetyllactosaminyl carbohydrate chains of band 3 on the cell surface (39). It has been shown that aggregation of the carbohydrate chains of band 3 glycoprotein is an important preceding event for removal of senescent erythrocytes from the circulation. The clustered carbohydrate chains generated by aging or oxidation are effectively recognized by anti-band 3 autoantibody (21,43) as well as by macrophages (44). It is not known what mechanism is involved in the oxidative aggregation of glycoproteins. Two mechanisms are possible. Firstly, aggregation may be caused by reaction of the lipid oxidation products with the peptide regions of the glycoproteins. This possibility is unlikely, however, because lipid peroxidation and protein aggregation of rat erythrocyte membranes induced by *in vitro* oxidation seemed to occur as independent events (45). The second possibility is that glycation and subsequent metal-dependent oxidation of senescent erythrocytes might be responsible for aggregation of glycoproteins including band 3 glycoprotein. In the circulation, erythrocyte membrane glycoproteins may react with glucose to form Amadori adducts, which might then be converted into CML and other AGE-structures. The presence of CML in membrane proteins of senescent erythrocytes demonstrated in the present study may suggest the possibility that aggregation of glycoproteins from senescent erythrocytes is caused by glycation and subsequent oxidation. Further studies are needed to elucidate the biological significance of formation of CML and other AGE-structures in senescent erythrocyte membranes.

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