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Research Article

Investigation of pathways of advanced glycation end-products accumulation in macrophages

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Advanced glycation end-products (AGE) play a role in the pathogenesis of several diseases, including diabetic complications and atherosclerosis. In atherosclerotic lesions of human aortas, AGE are localized in the extracellular matrix and intracellularly in foam cells. Two interpretations are possible for AGE accumulation inside macrophages, one is endocytic uptake of extracellular AGE-proteins by scavenger receptors; the other is intracellular AGE formation inside the macrophages. In the present study, we determined the pathways involved in AGE accumulation inside macrophages. RAW 264.7 cells, a murine macrophage cell line, incubated with BSA and 1600 mM glucose for 40 weeks, recognized heavily modified AGE- BSA. In contrast, the cells showed no ligand activity for mildly modified AGE-BSA, prepared by incubating BSA with 50 mM glucose for 24 weeks. Nε-(carboxymethyl)-lysine (CML)-modified proteins of about 65 kDa were detected in human monocyte-derived macrophages incubated for 7 days with 30 mM glucose and phorbol myristate acetate. Furthermore, CML was generated when glycated protein was incubated with hypochloric acid. Taken together, our results indicate that AGE detected inside foam cells in atherosclerotic lesions are generated intracellularly rather than representing endocytic uptake of extracellular AGE-proteins by scavenger receptors.

Keywords: Advanced glycation end-product / Nε-(carboxymethyl)lysine / Macrophage / Scavenger receptor

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1 Introduction

Glucose and other aldehydes, such as glyoxal, methylglyoxal and glycolaldehyde, react with amino residues of proteins to form Schiff base and Amadori products. Further incubation converts these early products into irreversible derivatives termed advanced glycation end-products (AGE). Cellular interaction with AGE-modified proteins is believed to induce several biological responses, which are involved in the development of diabetic vascular complications [1]. These cellular interactions are thought to be mediated by AGE receptors such as SR-A (class A scavenger receptor types I and II) [2, 3], CD36 [4], SR-BI (scavenger receptor class B type-I) [5], LOX-1 (lectin-like Ox-LDL receptor-1) [6], HA-SR (hyaluronan scavenger receptor) (same molecule as FEEL-1) [7, 8] and RAGE (receptor for AGE) [9, 10]. We reported previously that SR-A, which is known as a receptor for oxidized low-density lipoprotein (Ox-LDL), plays a role in the endocytic uptake and lysosomal degradation of AGE- BSA by macrophages [11]. AGE accumulate in human atherosclerotic lesions and interaction between AGE-modified proteins and scavenger receptor(s) expressed on macrophages and smooth muscle cells is known to induce the production of several cytokines such as plasminogen activator [12] and transforming growth factor-beta [13]. Furthermore, heavily modified glycolaldehyde-derived AGE-LDL induces foam cell formation from macrophages [14]. Considered together, these studies suggest that AGE-modified proteins and lipoproteins are involved in the pathogenesis of atherosclerosis. Therefore, attempts are currently underway to develop competitive inhibitors of the scavenger receptor-AGE proteins interaction that can effectively inhibit the development of athero-

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Abbreviations: AGE, advanced glycation end-product(s); **CML**, Nε-(carboxymethyl)lysine; **hm-AGE-BSA**, heavily-modified AGE-BSA; **HOCl**, hypochloric acid; **HRP**, horseradish peroxidase; **mm-AGE-BSA**, mildly-modified AGE-BSA; **PMA**, phorbol myristate acetate; **TCA**, trichloroacetic acid

sclerosis. In contrast to the endocytic pathway of AGE-proteins through AGE receptors, Brownlee and colleagues [15, 16] reported that incubation of fetal bovine aortic endothelial cells with 30 mM glucose for up to 7 days resulted in intracellular generation of AGE-proteins, which were detected by dot blotting using anti-N^{ε}-(carboxymethyl)lysine (CML) mAb (6D12). Taken together, the above studies suggest that AGE-proteins could accumulate within macrophages and endothelial cells by either endocytic uptake of AGE-proteins through AGE-receptor(s) or the formation of AGE-proteins intracellularly.

The present study was designed to determine which of the above pathway is most likely involved in accumulation of AGE-proteins within macrophages. We measured the effect of hypochloric acid (HOCl) on CML formation from Amadori products, because HOCl, generated from myeloperoxidase system, is known to produce reactive oxygen species by macrophages and neutrophils that kill invading bacteria.

2 Materials and methods

2.1 Chemicals

D-glucose, BSA and fatty acid-free BSA were purchased from Wako (Osaka, Japan). Tissue culture medium was from Gibco BRL (Grand Island, NY). Na ^{125}I was obtained from Amersham Biosciences (Arlington Heights, IL). All other chemicals were of the best grade available from commercial sources.

2.2 Preparation of AGE-modified BSA

Heavily modified AGE-BSA (hm-AGE-BSA) was prepared as described previously [13]. Briefly, 0.2 g/mL of fatty acid-free BSA was dissolved in 0.5 M sodium phosphate buffer (pH 7.4) with 1.6 M of D-glucose, sterilized by ultrafiltration and incubated at 37°C for 40 weeks, followed by dialysis against PBS. Mildly modified AGE-BSA (mm-AGE-BSA) was prepared by incubating 0.05 g/mL of fatty acid-free BSA with 50 mM of glucose in 0.05 M sodium phosphate buffer (pH 7.4) at 37°C for 24 weeks, followed by dialysis against PBS. Lysine and CML contents in the samples were quantified by amino acid analysis after acid hydrolysis with 6 N HCl for 24 h at 110°C, as described previously [17].

2.3 Preparation of glycated HSA and reduction

Glycated HSA, used as an Amadori protein, was prepared under antioxidative conditions that inhibit AGE formation as described previously [18]. Briefly, 50 mg/mL of HSA 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), followed by dialysis against 50 mM sodium phosphate buffer (pH 7.2). Reduction of Amadori product was conducted with a final concentration of 100 mM sodium borohydride in 0.25 M borate buffer (pH 9.2) for 4 h at room temperature.

2.4 Incubation of glycated HSA with HOCl

Glycated HSA (2 mg/mL) was incubated with HOCl (1 mM) at ambient temperature for 5 s, followed by determination of CML by noncompetitive ELISA.

2.5 ELISA

ELISA was performed as described previously [19]. Briefly, each well of a 96-well microtiter plate was coated with 100 μL of the sample to be tested in PBS, blocked with 0.5% gelatin, and washed three times with PBS containing 0.05% Tween 20 (washing buffer). Wells were incubated with 0.1 mL of 6D12 (0.1 μg/mL) dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by addition of 0.1 mL of 1 M sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader. Ikeda *et al*. [20] reported that 6D12 significantly recognizes CML and CML-protein adduct and its reactivity correlated with CML content, which was determined by HPLC. Furthermore, we demonstrated previously that CML is detected by 6D12 in peroxynitrite-treated glycated HSA [18].

2.6 Cellular assays

Modified BSA preparations were radiolabeled with ^{125}I using Iodo-Gen (Pierce, Rockford, IL) and dialyzed against PBS. RAW 264.7 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (medium A). For the uptake experiments, 2.5×10^5 RAW 264.7 cells were seeded in each well of a 24-well culture plate (15.5-mm diameter, Corning, Medfield, MA) in 1.0 mL of medium A and cultured for 24 h to subconfluence. The cells were washed with 1.0 mL of PBS and re-incubated with DMEM containing 3% BSA, 100 U/mL penicillin and 100 μg/mL streptomycin (medium B). The cells in each well were incubated at 37°C for 8 h in 0.5 mL of medium B with three different concentrations of ^{125}I-hm-AGE-BSA or ^{125}I-mm-AGE-BSA, in the presence or absence of their 50-fold unlabeled ligands. After 8-h incubation, 0.375 mL of the culture medium was taken from each well and mixed with 0.15 mL of 40% trichloroacetic acid (TCA) in a vortex mixer. To this solution, we added 0.1 mL of 0.7 M $AgNO_3$, followed by centrifugation. The resultant supernatant (0.25 mL) was

used to determine TCA-soluble radioactivity, which was considered as an index of cellular degradation. To measure cell-associated radioactivity, each well was washed twice with 1.0 mL of ice-cold PBS containing 1% BSA and two more times with ice-cold PBS. The cells were lysed with 1.0 mL of 0.1 N sodium hydroxide for 1 h at 37°C to determine the cell-bound radioactivity and cellular proteins. The protein concentration was measured by bicinchoninic acid protein assay reagent (Pierce). The radioactivity of the sample was determined by gamma counter (ARC 360, Aloka, Tokyo, Japan). The amount of cell-associated and degraded AGE-BSA was measured based on the specific radioactivity of hm- and mm-AGE-BSA; 922 and 682 cpm/ng, respectively. Specific association and degradation were determined by subtracting nonspecific value from the total value [3].

2.7 Human monocyte-derived macrophages

Human peripheral mononuclear cells were isolated from blood of healthy volunteers by the Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences). Purified monocytes were suspended in RPMI 1640 at 2×10^6 cells/mL and seeded onto 6-cm dishes (2×10^6) or 10-cm dishes (1×10^7) (Becton Dickinson Falcon PRIMARIA, Tokyo). After incubation for 1 h for adherence, the medium was replaced with RPMI 1640, with the indicated glucose or mannitol concentration and supplemented with 10% pooled human serum, streptomycin (0.1 mg/mL) and penicillin G (100 U/mL). Cells were incubated for 7 days to differentiate into macrophages.

2.8 Immunoblot analysis

Human monocyte-derived macrophages were solubilized with 1% Triton X-100, and the protein concentration determined using the BCA protein assay reagent, followed by pretreatment with boiling for 3 min in 2% SDS and 2-mercaptoethanol.

These samples were run on 10% gradient SDS-polyacrylamide gels, followed by electrophoretic transfer to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were exposed to polyclonal anti-CML antibody [15] and visualized by horseradish peroxidase-conjugated anti-rabbit IgG antibody with ECL Western blotting detection reagent (Amersham Biosciences). CML-proteins were detected by Western blotting, using CML-HSA as a positive control.

2.9 Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. A p value less than 0.05 denoted the presence of a statistically significant difference.

3 Results and discussion

Modification of proteins by reactive aldehydes is thought to play a role in the pathogenesis of several diseases, including diabetes and atherosclerosis. We demonstrated previously that AGE-modified proteins [3] and AGE-LDL [14] are recognized by SR-A, and may contribute to the pathogenesis of atherosclerosis. In the present study, we compared ligand activity of RAW 264.7 cells incorporated with hm- and mm-AGE-BSA. The extent of lysine modification was 71% (42 out of 59 lysines were modified) for hm-AGE-BSA and 23% for mm-AGE-BSA. Furthermore, CML contents for hm- and mm-AGE-BSA were 8.2 mol CML/mol HSA and 1.1 mol CML/mol HSA, respectively. When RAW 264.7 cells were incubated with ^{125}I-labeled ligand at three different concentrations at 37°C for 8 h, a significant amount of ^{125}I-hm-AGE-BSA was associated with these cells and the association was dose-dependent (Fig. 1A) and subjected to endocytic degradation by the same cells (Fig. 1B), whereas these changes were not observed in ^{125}I-

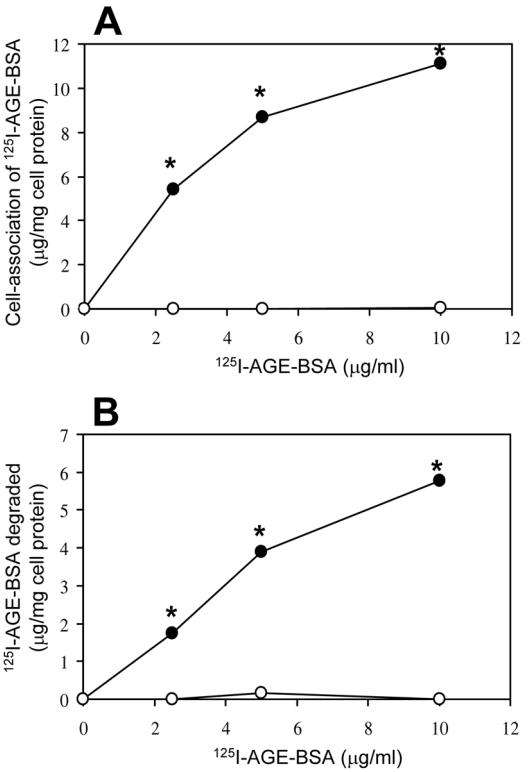

Figure 1. Endocytic uptake and subsequent degradation of AGE-BSA in RAW 264.7 cells. Cells were incubated at 37°C for 8 h with the indicated concentration of ^{125}I-hm-AGE-BSA (closed circles) or ^{125}I-mm-AGE-BSA (open circle) in the presence or absence of their 50-fold unlabeled ligands. The cells-specific association (A) and degradation (B) of ^{125}I-hm-AGE-BSA and ^{125}I-mm-AGE-BSA were calculated by correcting for nonspecific cell association and degradation. Data represent the mean values of two separate experiments. *, $p < 0.05$, compared with the respective values of ^{125}I-mm-AGE-BSA.

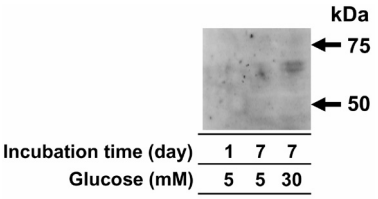

Figure 2. Generation of CML during maturation of macrophages. Human monocytes were incubated for 7 days with the indicated glucose concentrations in the presence of PMA to differentiate into macrophages. CML-proteins were detected by polyclonal anti-CML antibody as described in Section 2. Experiments were repeated twice with almost identical results.

mm-AGE-BSA (Figs. 1A and B). These data demonstrated that mm-AGE-BSA is not recognized by AGE receptor(s) expressed on RAW 264.7 cells. Since scavenger receptors, such as SR-A, CD-36, LOX-1 and SR-BI, are expressed on macrophages whereas RAGE is expressed on endothelial cells, it is conceivable that hm-AGE-BSA could be recognized by scavenger receptors expressed on RAW 264.7 cells.

Thornalley *et al.* [21] demonstrated that end-stage renal disease is associated with a significant increase in the molecular mass of HSA (+255 Da, relative to control subjects). However, our study using MALDI TOF mass analysis demonstrated that the molecular mass of mm-AGE-BSA was 658 Da larger than native BSA (data not shown), indicating that our experimentally prepared mm-AGE-BSA is already more profoundly modified than physiological HSA under (patho)physiological conditions. These results indicate that endocytic uptake of AGE-proteins through scavenger receptors is negligible or unlikely to occur *in vivo*.

With regard to intracellular AGE formation, Nishikawa *et al*. [16] demonstrated that intracellular AGE content, which was determined by dot blotting using 6D12, increased when fetal bovine aortic endothelial cells were incubated with 30 mM glucose for 5 days. Therefore, we investigated whether AGE are generated upon cell injury. For this purpose, human monocytes were incubated with 5 or 30 mM glucose for 1 or 7 days in the presence of 100 nM of phorbol myristate acetate (PMA, for maturation of these cells to macrophages) and then analyzed the presence of CML-protein in these cells by Western blotting. As shown in Fig. 2, 65.0- and 65.3-kDa bands, corresponding to CML-modified proteins, were observed by immunochemical analysis after 7 days of incubation with 30 mM glucose, whereas 25 mM mannitol, used as an osmolarity control, in the presence of 5 mM glucose did not increase CML (data not shown). These results suggest that exposure of macrophages to high glucose levels injurious to the cells, could result in the generation of CML-modified proteins, and that AGE detected inside macrophages in atherosclerotic lesions are generated intracellularly.

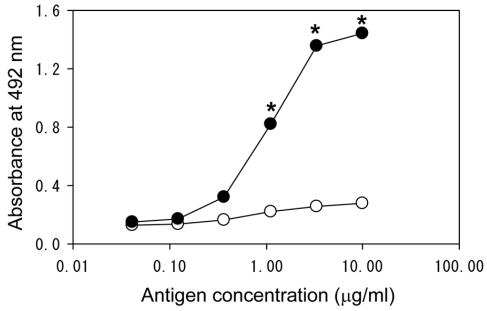

Figure 3. Effects of hypochloric acid on CML formation. Glycated HSA (2 mg/mL) was incubated with HOCl (1 mM) at ambient temperature for 5 s, and CML content of HOCl-treated (closed circles) and untreated glycated-HSA (open circles) was determined by non-competitive ELISA. Experiments were repeated twice with almost identical results. *, $p < 0.05$, compared with the values of untreated glycated-HSA.

In the next step, we assessed the effect of HOCl on CML formation from Amadori product since HOCl is generated from myeloperoxidase system in neutrophils and macrophages and known to oxidize proteins. Glycated HSA was incubated with HOCl at ambient temperature for 5 s, followed by determination of CML by non-competitive ELISA. As shown in Fig. 3, a significant amount of CML was formed by HOCl treatment, whereas CML was at background levels in experiments with glycated HSA without HOCl. Furthermore, CML content did not change when reduced-glycated HSA was incubated with 10 mM HOCl (data not shown).

Hammes *et al*. [22] reported that thiamine and benfotiamine prevent intracellular AGE formation by reducing the concentration of methylglyoxal, a strong AGE-precursor, and hence inhibit diabetic retinopathy. Furthermore, Babaei-Jadidi *et al*. [23] demonstrated that administration of thiamine and benfotiamine resulted in reduction of intracellular methylglyoxal concentration by increasing transketolase expression and prevented the development of diabetic nephropathy in diabetic rats. Taken together, these findings suggest that compounds that inhibit intracellular AGE formation in macrophages could be potentially useful agents for the treatment of diabetic complications and atherosclerosis compared with competitive inhibitors for scavenger receptors.

Figure 4 depicts the likely mechanism of CML formation. A carbon chain between C-2 and C-3 of dicarbonyl, which has been converted from an Amadori compound through enediol [24], is cleaved by HOCl, followed by formation of CML. Although a significant amount of CML formation was detected in glycated-HSA after HOCl treatment (Fig. 3), there are other pathways for generation of reactive oxygen species in macrophages such as hydroxyl radical and peroxynitrite, and they are also known to contribute to CML formation [25, 18] (Fig. 4).

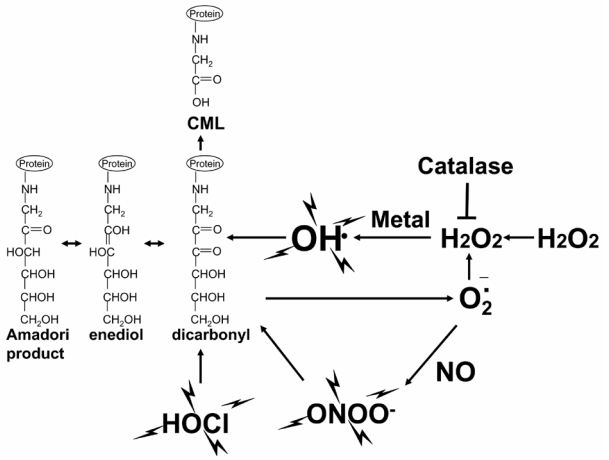

Figure 4. Possible pathways of CML formation.

4 Concluding remarks

Further studies are required to elucidate the pathways of CML formation inside macrophages. Such research could help design new strategies for treatment of diabetic complications and atherosclerosis.

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