ADVANCED GLYCOSYLATION ENDPRODUCTS ON ERYTHROCYTE CELL SURFACE INDUCE RECEPTOR-MEDIATED PHAGOCYTOSIS BY MACROPHAGES

A Model for Turnover of Aging Cells

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Recent studies (1–3) have indicated that glucose can react nonenzymatically with amino groups of a wide range of proteins throughout the body, including intracellular proteins such as hemoglobin and lens crystallins, extracellular proteins such as collagen, and cell membrane proteins on red cells (1) and endothelial cells (2, 3). Initially a reversible adduct, the Amadori product is formed from the nonenzymatic reaction of glucose with proteins (4, 5). However, these Amadori products can slowly undergo a series of further reactions and rearrangements that give rise to complex irreversible protein adducts, advanced glycosylation endproducts (AGE). These AGE continue to accumulate in extracellular and membrane proteins as a function of time and glucose concentration (2, 6).

Recently, a new membrane-associated macrophage receptor has been identified that recognizes proteins modified by this process of long-term nonenzymatic AGE formation (7, 8). This receptor has been shown not to recognize low-density lipoprotein (LDL), acetyl-LDL, mannose/fucose, and formaldehyde-treated proteins (9). Because formation of AGE increases with protein age (9–11) and AGE receptor–mediated uptake and degradation of these proteins increases with amount of AGE formation (7), it has been postulated (7, 8) that the AGE receptor preferentially mediates the removal of senescent macromolecules. In disorders such as diabetes mellitus, in which accelerated AGE formation occurs (2), the efficiency of such a removal mechanism may influence the rate at which diabetic tissue damage develops.

Reasoning that certain cells have a long enough lifespan to allow AGE formation on their surface proteins, we hypothesized that the AGE receptor might also mediate the removal of aging cells by selectively recognizing a cell-surface alteration that accumulates over time. This mechanism would also account for

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1 Abbreviations used in this paper: AGE, advanced glycosylation endproducts; CMC, 1-cyclohexyl-3-[2-(4-morpholinyl)-ethyl]-carbodiimide; FFI-HA, [3-furoyl-4-(5)-[2-furany]-1H-imidazole]-hexanoic acid; G-6-P, glucose-6-phosphate; LDL, low-density lipoprotein.
the increased removal rate of red cells observed in diabetes, where prolonged hyperglycemia is associated with accelerated AGE formation and shortened red cell survival (11). In this report, we describe a series of experiments in which intact cell recognition and binding by the AGE receptor was evaluated, using the red blood cell as a model.

Materials and Methods

*RBC Preparation.* Human blood (2.0 ml) from normal, healthy adult volunteers was collected in heparinized tubes. After removal of plasma and buffy coat, the RBCs were washed four times with 10 vol of Ca²⁺- and Mg²⁺-free PBS, pH 7.4, and were resuspended in DMEM.

BALB/cj mice (The Jackson Laboratory, Bar Harbor, ME) were rendered diabetic by intraperitoneal injection of freshly prepared alloxan monohydrate (40 mg/kg) dissolved in 0.1 M sodium phosphate buffer (pH 3.0). The presence of diabetes was assessed by measuring serum glucose concentrations with a glucose analyzer (Bio-Dynamics, Indianapolis, IN) 48 h after injection and at weekly intervals thereafter. All diabetic animals had plasma glucose concentrations >300 mg/dl. 3 mo after alloxan administration, mouse erythrocytes were collected in heparinized tubes and prepared as described above for human RBC.

*Opsonized RBC Preparation.* 0.1 ml of a 15% RBC suspension from a D⁺ donor was added to 0.5 ml of a high-titer anti-D serum. After incubation at 37°C for 30 min, the cells were washed three times with PBS and resuspended in 3 ml of DMEM.

*AGE-RBC Preparation.* The specific AGE [2-furoyl-4(5)-(2-furanyl)-1H-imidazole]-hexanoic acid (FFI-HA) was synthesized as described previously (12). In brief, furylglyoxal hydrate (10 mmol) in 3:1 dioxane/water was treated with 6-aminohexanoic acid (15 mmol) and triethylamine (15 mmol) and stirred at 25°C for 1 h. After the addition of concentrated aqueous ammonia, the mixture was diluted with 5% NaH₂PO₄ and extracted with CH₂Cl₂, washed with brine and filtered through active carbon and MgSO₄. The crude product was purified by medium pressure chromatography on silica gel yielding FFI-HA as straw-colored flakes (melting point 105-106°C).

Freshly washed normal RBCs were resuspended in PBS, with and without 10 mM of the water-soluble carbodiimide, 1-cyclohexyl-3-[2-(4-morpholinyl)-ethyl]-carbodiimide (CMC) (12), to which FFI-HA was added at different concentrations (10-100 μM). The mixtures were incubated with continuous mixing for 1 h at room temperature. Parallel mixtures containing RBCs alone in PBS or RBCs and carbodiimide (10 mM) were treated identically as controls. After three washes, the cells were resuspended in 1 mM glycine in PBS and left to incubate for 30 min. After three washings with PBS, the cells were suspended in RPMI 1640 before phagocytosis assay.

*Glycosylated RBC Preparation.* To produce nonenzymatically glycosylated erythrocytes, glucose, glucose-6-phosphate (G-6-P), xylose, arabinose, fructose, and galactose were added to freshly isolated normal human red cells suspended in DMEM at a concentration of 100 mM and incubated for 48 h at room temperature. RBC suspensions without added sugars were used as control cells. After incubation, the cells were washed three times with PBS and were suspended in RPMI 1640.

AGE-BSA was prepared by incubating BSA in 50 mM glucose at 37°C for 6 wk, in the presence of protease inhibitors (PMSF, 1.5 mM; EDTA, 0.5 mM) and antibiotics (penicillin, 100 U/ml; gentamicin, 40 mg/ml) as described previously (7, 8).

To determine the amount of FFI bound to red cell membrane protein and membrane-bound FFI, erythrocyte ghosts from FFI-coupled RBCs, as well as from the various control and sugar-incubated RBCs were prepared according to the method of Dodge et al. (14) and were stored at 4°C in 5 mM sodium phosphate buffer, pH 8.0. Protein determinations were performed according to the method of Bradford et al. (15) after solubilizing aliquots of ghost RBCs in 1 N NaOH. Membrane-bound FFI was determined by radioimmunoassay, as described previously (16). FFI-RBC contained 571 pmol/mg membrane protein; opsonized RBC, 7.1 pmol/mg; and PBS-RBC, 8.9 pmol/mg of membrane protein.
Glucose-RBC contained 168 pmol/mg; G-6-P-RBC, 209 pmol/mg; xylose-RBC, 240 pmol/mg; and arabinose-RBC, 280 pmol/mg of membrane protein.

**Human Monocyte Preparation.** The buffy coat from 100 ml fresh human blood was diluted twofold with saline containing 1 mM EDTA, pH 7.4, and mononuclear cells were separated from other elements of the blood by centrifugation on Ficoll-Paque gradients as described before (17). The mononuclear cells were washed three times in cold RPMI 1640 (Gibco, Grand Island, NY) to remove platelets, and the cells were suspended in RPMI 1640 made 10% in normal human serum. The monocytes were further purified by the Percoll method (18, 19). Percoll was brought to isotonocity by the addition of 0.1 vol of 10× PBS. 1 ml of normal human serum, 14.7 ml PBS, and 22 ml isotonic Percoll was mixed in sterile 50-ml centrifuge tubes and centrifuged for 25 min at 8,000 g at 5°C in a Sorvall SS-34 rotor. 5 ml of the mononuclear suspension were layered on the resulting gradients, and the tubes were centrifuged at 1,500 g for 25 min at 5°C in a swinging-bucket rotor. The resulting bands were detected by light scattering, and band I, corresponding to monocytes, was transferred and cultured in screw-cap Teflon jars (Savillex, Minnetoka, MN), at 10⁶ cells/ml in RPMI 1640 with 12.5% human serum at 37°C in 5% CO₂ for 5–7 d. Cell viability was assessed by trypan blue exclusion. Plating efficiency of phagocytes on a tissue-culture plastic surface was measured before and after attachment. >99% of the adhering cells were monocytes, as assessed by their ability to spread on surfaces (19) and ingest IgG-coated erythrocytes (20). When ready for use, aliquots of 10⁶ monocytes were plated in sterile petri dishes containing precleaned round coverslips, and incubated for 2 h at 37°C in 5% CO₂. After the appropriate washes, ~7.0 × 10⁵ cells adhered routinely.

**Phagocytosis Assay.** Before the addition of RBCs, the monocyte cultures were washed twice with RPMI 1640. The various red cell suspensions were added to each well to give a 100-fold excess of red cells to monocytes, and the cells were then incubated at 37°C for up to 2 h. At the indicated times the coverslips were removed from the wells, washed three times with RPMI to remove nonadherent material, placed into clean wells, and fixed with 1.25% glutaraldehyde in PBS for 30 min. Duplicate coverslips were counted using 40× phase microscopy. At least 300 monocytes from three or more randomly selected fields were counted per well, and duplicate wells were evaluated for each data point. The data were expressed as the number of positive monocytes (monocytes with erythrocytes ingested) per 100 monocytes (21, 22). Ingestion index, the number of ingested red cells per positive monocyte, was also determined (23).

**Mouse Peritoneal Macrophage Preparation.** Resident mouse peritoneal macrophages were isolated from BALB/c female mice as described previously (21, 23), and plated in wells containing glass coverslips as described above for human monocytes. The phagocytosis of mouse red cells from normal and diabetic BALB/c mice by syngeneic macrophages was performed identically with that described above for human RBC. Inhibition experiments were carried out in the absence and presence of saturating concentrations of AGE-BSA (0.1–1.0 mg/ml of medium) (7–9). Opsonized human RBC prepared as described above were used as controls. In these experiments the data are expressed as percentage of macrophages having five or more ingested RBC per macrophage.

**FFI-RBC Half-life Assay.** BALB/c inbred mice were bled by cardiac puncture, yielding ~2.0 ml of blood. The red cells were washed with 10 vol Ca²⁺- and Mg²⁺-free PBS and coupled with FFI in the presence of 10 mM CMC as described above. RBCs incubated in PBS with and without CMC were used as controls. Subsequently all cell suspensions were labeled with ⁵¹Cr by adding 0.2 mCi NaCl⁵¹CrO₄ (New England Nuclear, Boston, MA) to 2 ml of 50% packed RBC in RPMI-1640 medium for 1 h at 37°C. The labeled cells were washed with PBS at least four times to remove unbound isotope. Three BALB/c mice were then injected intravenously with 200 μl of each of the four types of RBC suspension, containing 7.5 × 10⁵ cpm. At the indicated times, mice were bled (0.05 ml) and level of radioactivity was determined.

**Statistics.** All data are expressed as mean ± SEM of six experiments. p values were obtained using Student’s unpaired t test.
Results

When 7-d-old cultured human monocytes were exposed for 2 h to FFI-coupled RBCs, a large number of monocytes (55 ± 6%) bound the modified red cells (Fig. 1). This number was significantly higher than the binding of control PBS-treated cells (4 ± 2%) ($p < 0.0025$) or cells that were incubated in carbodiimide alone (6 ± 0.9%) ($p < 0.0025$) and almost as high as that observed with IgG-coated red cells (70 ± 9%). Similarly, the phagocytic index of FFI-treated RBCs was significantly greater (3.4 ± 0.8) than both normal control (1.2 ± 0.2) ($p < 0.0025$) and carbodiimide-treated cells (1.5 ± 0.2) ($p < 0.0025$), but less than that of IgG-coated RBCs (4.9 ± 1.6).

To differentiate surface-attached from ingested erythrocytes, a hypotonic solution (PBS diluted 1:4 in water) was added to selected wells for 10 s, followed by the addition of fixative. Maximal monocyte endocytosis of FFI-RBC was complete within 60 min, in contrast to opsonized cells, which were maximally ingested within 30 min.

Exposure of FFI-coupled RBCs to monocytes cultured for 1–9 d revealed that maximal uptake of FFI-coupled red cells occured on day 7 (Fig. 2). Significant uptake was noted on day 1, followed by a rapid decline over the next 48–96 h and a subsequent increase of uptake from days 3 to 7. The uptake of opsonized cells was uniform throughout the entire incubation period.

To establish the specificity of the interaction of FFI-RBCs with human monocytes, competition experiments were carried out in which binding and ingestion of red cells were observed in the presence and absence of nonenzymatically glycosylated BSA (AGE-BSA), prepared as described in Materials and Methods (12). As shown in Fig. 3, the addition of AGE-BSA at a concentration of 500 μg/ml inhibited monocyte binding of FFI-RBCs by >70% and binding of normal PBS-treated RBCs by 10–20%. In contrast, AGE-BSA did not inhibit IgG-RBC. Similar results were observed with 1.0 mg/ml of inhibitor (data not shown).
FIGURE 2. Effect of in vitro culture of normal human monocytes on binding of FFI-RBC (dark circles), prepared as described in Materials and Methods. IgG-RBC (open circles) and PBS-RBC (open triangles) were used as controls. Data, representing the mean ± SEM of three different experiments, each performed in duplicate, are expressed as percent of monocytes with ingested erythrocytes.

FIGURE 3. Competitive inhibition of FFI-RBC binding by normal 7-d-old human monocytes by excess AGE-BSA (dark bars). IgG-RBC and PBS-RBC were used as controls. Data, representing the mean ± SEM of six different experiments, each performed in duplicate, are expressed as percent of monocytes with ingested erythrocytes. 100% values were FFI-RBC 55 ± 6, IgG-RBC 70 ± 9, and PBS-RBC 4 ± 2.

These data suggested that FFI-modified red cells are recognized and bound specifically by the monocyte AGE-binding site.

We subsequently induced accelerated AGE formation on red cell surface proteins by a 48-h, 25°C incubation of human RBC with different sugars, (glucose, G-6-P, xylose, arabinose, galactose, and fructose) at 100 mM concentrations. At the end of this period, the media demonstrated no evidence of cell lysis, and red cells appeared microscopically indistinguishable from the controls. As demonstrated by RIA (Materials and Methods section), red blood cells incubated in the presence of high glucose and G-6-P concentrations underwent formation of a significant amount of AGE on their cell membrane proteins. Uptake of RBCs from each group was determined by the human monocyte phagocytosis assay. As indicated in Fig. 4, 15% of the monocytes bound glucose-incubated RBCs and 26% bound G6P-treated RBCs, as compared to 6% of monocytes binding control PBS-RBCs. Similarly, fructose- and galactose-incubated RBCs bound to 12.5 and 16.5% of the monocytes, respectively. A much lower number of monocytes bound red cells that had been preincubated with either xylose or arabinose (7.5% for each).

When in vivo glucose-modified diabetic mouse RBCs were added to normal mouse macrophages, a significant increase in phagocytosis was observed in comparison to that of normal red cells (21.5 vs. 8.2%, \( p < 0.005 \)) (Fig. 5A). Competition with excess AGE-BSA revealed inhibition of diabetic red cell ingestion by 70%, and inhibition of normal red cell ingestion by 25%. In contrast, in
similar experiments with opsonized erythrocytes, AGE-BSA failed to compete even at maximal AGE-BSA concentrations (1 mg/ml) (Fig. 5B). To determine whether autologous red blood cells modified by an AGE such as FFI-HA are recognized by macrophages in vivo and removed from the circulation faster than nonmodified cells, BALB/c inbred mouse red cells were incubated either with FFI-HA (as described in Materials and Methods), or with PBS alone for 1 h at room temperature. As an additional control, mouse cells were treated with carbodiimide alone (10 mM). After $^{51}$Cr-labeling, all three groups of cells were reinjected intravenously into syngeneic mice and erythrocyte radioactivity was monitored for 20 d. Within the first h after injection, ~9% of the initial radioactivity was found in the serum fraction of all three groups. This was
presumably due to traumatic hemolysis caused by ex vivo handling, since it was reduced to 0.4% in all three groups within the first 24 h. As shown in Fig. 6, the half-life of FFI-treated red cells was observed to be 7 d, compared to the control cell half-life of 20 d. This value for the control cells is in agreement with previous studies in mice using the same method (24). Cells treated with carbodiimide alone had a nearly normal in vivo half-life.

Discussion

In this report we have extended our previous observations on the recognition of AGE on proteins by a specific monocyte/macrophage receptor. We now present evidence that both synthetic and naturally formed AGE adducts attached to the surface of intact human cells induce binding and ingestion of these cells by normal human monocytes. The recognition and removal of these modified cells appears to be mediated by the specific macrophage AGE receptor, since AGE-modified red cell binding is specifically inhibited by competitive binding of AGE-BSA (7–9). We further demonstrate that the presence of AGE on the red cell surface in amounts significantly greater than found on unmodified cells leads to a shortened erythrocyte survival in vivo, presumably due to the more rapid removal of these AGE-cells by splenic and hepatic phagocytic cells.

Red cells were selected as an appropriate model for two reasons. First, erythrocyte membrane proteins are synthesized during erythropoiesis and remain intact, with negligible turnover, throughout the cell’s 3–4 mo lifespan (1, 25). These proteins thus survive long enough for AGE formation to occur. Second, enhanced Amadori product formation has been demonstrated previously on diabetic red cell membrane proteins (1) in association with shortened red cell survival in vivo (26).

Nonenzymatically glycosylated proteins normally undergo a slow series of further reactions and rearrangements leading to the formation of irreversible AGEs that accumulate with protein age (4). These accumulating AGEs are specifically recognized by the macrophage AGE receptor (7), and thus appear to function as a time-dependent signal for the removal of senescent macromolecules. Because human red blood cells survive for a prolonged period of time, it was hypothesized that the increasing amounts of AGE, which would form progressively on their surface, would trigger removal from the circulation via the specific
AGE receptor system. The results of the above studies are consistent with this hypothesis.

Nonenzymatic glycosylation of erythrocyte membrane proteins has been shown previously (1) to be extensive and relatively nonspecific, occurring primarily at the lysine residues of most major protein bands. In this report, we demonstrate that AGE are also present on normal intact red blood cells, and that AGE accumulation can be accelerated by exposure to high glucose levels, which in turn leads to increased monocyte AGE receptor binding and ingestion of glucose-modified red cells. Values obtained for red cell membrane AGE content may be higher than amounts available to interact with the macrophage AGE receptor in vivo, since the RIA method used in these studies for the measurement of AGE involves enzymatic hydrolysis of AGE-proteins (16). This may expose AGE adducts that are normally hidden.

Although the in vitro glucose concentration used in these experiments (100 mM sugar) was nonphysiologic, it was chosen to accelerate the formation of reactive AGE over the short, experimentally practical incubation period used, since 2-3 d of albumin incubation with the same glucose concentration in vitro has been shown previously (27) to result in detectable AGE formation. Because it is the integral of glucose concentration over time that determines the extent of AGE accumulation (2), this process occurs over a much longer period in vivo (10, 27-29), where concentrations of glucose found even in diabetic plasma rarely exceed 20 mM. After 3 mo of exposure to hyperglycemia, however, RBCs from diabetic mice showed a twofold increase in ingestion by mouse macrophages through the AGE receptor.

The increased AGE formation and binding of erythrocytes to monocytes after incubation with G-6-P compared to glucose, fructose, and galactose, reflects the ability of G-6-P to more readily form AGE, due to a higher percentage of reactive open-ring G-6-P molecules at equilibrium (24). The pentoses, xylose, and arabinose also react with protein amino groups faster than glucose to form a series of pigmented glycosylation products of undetermined structure (30). Surprisingly, however, these products did not induce binding and uptake by macrophages above that of normal control cells, despite an increased amount of FFI-like material, as measured by RIA. Evidently, the anti-FFI polyclonal antibody used in our assay recognizes and crossreacts with FFI-like products in these arabinose and xylose reactions. The AGE-receptor, on the other hand, appears to be more restrictive in its structural requirements. This phenomenon warrants further investigation, since the formation of different AGE with structures not recognizable by the AGE receptor or other scavenger monocyte receptors may result in accumulation of unremoved proteins in vivo.

In addition to increasing red cell uptake through interaction with the AGE receptor, accumulation of AGE on proteins of aging normal and diabetic red blood cells may also contribute to the removal of senescent erythrocytes by other receptor-mediated mechanisms as well. Nonenzymatic glycosylation results in increased crosslinking of proteins by facilitating formation of both disulfide bonds and glucose-derived, FFI-like crosslinks (4, 27, 31). In red cells, such age-related, glycosylation-dependent crosslinking (6, 31) may be responsible for the clustering of membrane proteins that is thought to be necessary for the appear-
ance of a unique senescent cell antigen with age (32–35). This antigen, which may be exposed by the process of clustering or which may arise de novo as a consequence of clustering, is then recognized by an IgG autoantibody that mediates subsequent recognition and uptake by macrophages (32). Similarly, the appearance of membrane glycoproteins recognizable by the N-acetyl-D-galactosamine receptor of hepatic Kupffer cells (36) could also arise by this mechanism.

Whether the senescent or glucose-modified cells of other tissues are eliminated via this mechanism is an important question that warrants further investigation, since removal-associated cellular damage could play a critical role in the development of tissue pathology in both aging and diabetes.

Summary

Glucose can react nonenzymatically with amino groups of proteins to form covalent Amadori products. With time these adducts undergo further rearrangements to form irreversible advanced glycosylation endproducts (AGE), which accumulate with protein age. A specific AGE, 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), has been identified on proteins in vivo. We have recently shown that a macrophage receptor specifically recognizes and internalizes proteins modified by AGE such as FFI, thus preferentially degrading senescent macromolecules.

Reasoning that cellular turnover may be mediated by macrophage recognition of AGE-membrane proteins, we prepared human RBCs with FFI attached chemically. Human monocytes were incubated with either FFI-RBCs, IgG-opsonized RBCs, or PBS-treated RBCs. Erythrophagocytosis of FFI-RBCs was significantly higher than that of PBS-RBCs (55 vs. 4%; p < 0.0025) and almost as high as that of IgG-RBCs (70%), and was competitively inhibited by AGE-BSA. AGE-RBCs were also prepared by incubating RBCs with various sugars. Human monocytes showed a 15% ingestion of glucose-RBCs, and a 26% ingestion of glucose-6-phosphate-RBCs, compared to 6% for PBS-RBCs. Similarly, diabetic mouse RBCs were phagocytosed by nearly three times more cells (21%) than normal mouse RBCs when exposed to syngeneic mouse macrophages. This phagocytosis was competitively inhibited (70%) by addition of excess AGE-BSA.

The in vivo half-life of $^{51}$Cr-labeled mouse FFI-RBCs injected into syngeneic mice was reduced to 7 d, as compared to a half-life of 20 d for the controls.

These data suggest that the macrophage receptor for the removal of glucose-modified proteins may also mediate the endocytosis of RBCs with AGE formed on their surface, and thus be responsible in part for the removal of some populations of aging cells.

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