Macrophage/monocyte-derived cells appear to play an important role in the regulation of extracellular matrix protein turnover, which is crucial to the maintenance of connective tissue homeostasis as organisms age (1, 2). Receptor-mediated endocytosis by macrophages and monocytes is also involved in the removal of deposited lipoproteins from arterial intima (3, 4). The physiologic ligand on selected macromolecules that is specifically recognized by macrophages in vivo has not yet been identified, however.

In vitro, covalent modification of low-density lipoprotein (LDL)¹ by chemical reagents such as acetic anhydride or maleic anhydride results in macrophage recognition and uptake of these modified proteins (5), while modification of albumin by treatment with formaldehyde results in similar recognition and uptake by hepatic phagocytes (6). Although no significant in vivo formation of either ligand has been demonstrated, specific, distinct receptors have been isolated and characterized for both acetyl-LDL and formaldehyde albumin (7, 8). The biological role of these receptors remains to be elucidated.

Recently (9, 10), a new membrane-associated macrophage receptor has been identified that recognizes proteins modified by a common in vivo process, long-term nonenzymatic reaction of glucose with proteins. This receptor specifically recognizes advanced glycosylation endproducts (AGE), which form slowly from initial Amadori glycosylation products through a series of further reactions, rearrangements, and dehydrations (11, 12). One of the AGE recognized by this receptor, 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole (FFI), is a condensation product of two glucose molecules and two lysine amino groups (13, 14). Scatchard analysis of AGE-protein binding data has indicated that there are ~10⁵ receptors per macrophage, with an affinity constant (Kₘ) of 1.75 × 10⁷/M (10). These AGE receptors are distinct from the mannose/fucose receptors involved in glycoprotein uptake (15), since yeast mannan does not compete with AGE-protein uptake by macrophages (10). However, the possibility that the AGE-protein receptor is

¹ Abbreviations used in this paper: AGE, advanced glycosylation end products; FFI, 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole; LDL, low-density lipoprotein.

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identical to other previously described scavenger receptors has not yet been excluded.

In this report, we describe competition and crosscompetition experiments between AGE-modified protein and a variety of previously described scavenger receptor ligands. Furthermore, the specific pattern of AGE-protein receptor inhibition by the polyanionic compounds polyinosinic acid, polyadenylic acid, polyglutamic acid, polycytidylic acid, fucoidin, and heparin was assessed and compared with that of acetyl-LDL. Similar experiments were performed using proteins modified by the specific AGE, FFI. The data obtained indicate that the macrophage receptor for glucose-modified proteins is distinct from previously described scavenger receptors.

Materials and Methods

AGE-BSA was prepared by incubating BSA in 50 mM glucose at 37°C and 4–6 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 (9). Aliquots of AGE-BSA were subjected to IEF and SDS-PAGE, and did not show any change in pI or Mw as compared with unmodified BSA. Radioiodination was carried out by the method of Fraker and Speck (16). FFI-BSA and FFI-LDL were prepared by covalently coupling FFI-hexanoic acid to BSA or freshly prepared human LDL using water soluble carbodi-imide, as described in detail previously (10, 17). Formaldehyde-treated albumin was prepared by dissolving BSA in 0.45 M sodium carbonate (pH 10.0), followed by centrifugation and the addition of formaldehyde solution (37% wt/vol) dropwise with stirring, as previously reported (18). After incubation at 37°C for 1 h, the solution was dialyzed overnight against 0.15 M NaCl, and then against distilled water at 4°C. After centrifugation at 20,000 g for 30 min at 4°C, the supernatant was ready for use. LDL was prepared from pooled normolipidemic human serum by density gradient ultracentrifugation in a vertical rotor (19, 20), and acetylated by repeated partial additions of acetic anhydride (Fischer Scientific Co., Springfield, NJ) for >1 h at 2°C, followed by dialysis against PBS (5). In all experiments, the same preparations of AGE-BSA, FFI-BSA, and acetyl-LDL were used. Maleyl-BSA was prepared by dissolving BSA in 0.2 M Na2B4O7. The pH was brought to 8.5, and the mixture was combined with an excess of solid maleic anhydride (Matheson, Coleman, and Bell, Norwood, OH) as previously described (5), until all acid was liberated, then dialyzed overnight. PBS-BSA and PBS-LDL refer to unmodified BSA and LDL in 0.1 M PBS.

Mouse peritoneal macrophages were isolated as described before (9) from female NCS mice (25–30 g) obtained from the Laboratory Animal Research Center facility of The Rockefeller University. In the competition studies described below, each well (10⁶ cells) received 10 μg (150 nM) radiolabeled AGE-BSA (sp act, 286 cpm/ng), 10 μg (150 nM) radiolabeled FFI-BSA (sp act, 358.1 cpm/ng), or 25 μg (63 nM) labeled acetyl-LDL (sp act, 272.0 cpm/ng) and the indicated amounts of unlabeled competitor. Competitors used in experiments with radiolabeled AGE-BSA and FFI-BSA were unmodified BSA, unmodified LDL, acetyl-LDL, maleyl-BSA, formaldehyde-treated BSA, fucoidin, polyinosinic acid, polyadenylic acid, polyglutamic acid, polycytidylic acid, and heparin sodium. After a 4-h incubation at 37°C, the amount of radiolabeled protein in the cells and the amount of 125I-labeled TCA-soluble material in the medium were determined in triplicate. Competition experiments with 131I-acetyl-LDL were performed in the presence of increasing concentrations of unlabeled acetyl-LDL, AGE-BSA, FFI-BSA, and FFI-LDL.

Results

Cellular uptake of radioiodinated AGE-BSA was not inhibited by the addition of increasing amounts of unlabeled BSA, while it was supressed to 10% of control.
by addition of unlabeled AGE-BSA (Fig. 1A). BSA chemically linked to the specific advanced glycosylation endproduct FFI also suppressed AGE-BSA uptake to ~20% of control. This suggests that the AGE receptor recognizes a specific type of AGE structure having important homology with FFI.

In contrast, addition of unlabeled formaldehyde-modified BSA (f-BSA) did not reduce cellular uptake of AGE-BSA (Fig. 1A), even at the highest concentration added (1.0 mg/ml). Cellular uptake of AGE-BSA was similarly unaffected by addition of increasing amounts of unmodified LDL. However, when competition experiments were performed using acetyl-LDL and maleyl-BSA, AGE-BSA uptake was suppressed to 23 and 25% of control values, respectively. Acetyl-BSA was totally ineffective in inhibiting AGE-BSA uptake by the cells (data not shown). The effects of these competitors on cellular degradation of AGE-BSA paralleled those observed with cellular uptake (Fig. 1B). Identical competition experiments performed using radiolabeled FFI-BSA in place of AGE-BSA gave identical results for both uptake and degradation (Fig. 2).

Although these data suggested that cellular uptake of AGE-BSA and uptake of acetyl-LDL and maleyl-BSA might be mediated by the same membrane receptor, it was equally likely that the observed competition reflected nonspecific membrane effects of these strong polyanionic competitors.

To distinguish between these two possibilities, competition experiments were performed using a series of highly negatively charged macromolecules, which included those previously shown (21) to selectively compete with acetyl-LDL for its binding site. The sulfated polysaccharide fucoidin strongly inhibits the binding of acetyl-LDL, for example, while other sulfated polysaccharides, such as heparin
and chondroitin sulfate A and C do not. In our experiments, fucoidin also inhibited the cellular binding of both AGE-BSA and FFI-BSA by >75% of control, with >50% inhibition observed at concentrations of 50 μg/ml (Fig. 3). However, unlike acetyl-LDL, binding of both AGE-BSA and FFI-BSA was inhibited at least to 50% of control by heparin. Comparison of the effects of a number of other polyanions on AGE-BSA binding to its receptor with those reported for acetyl-LDL revealed additional differences. Although both receptors were inhibited by polyinosinic acid and not by polycytidylic or polyglutamic acid, only binding to the AGE-BSA receptor was inhibited by polyadenylic acid.

These results suggested that the AGE-protein receptor, like several other scavenger receptors, is sensitive to the effects of polyanionic compounds. However, the specificity of inhibition by these compounds is distinct from that which has been previously described for receptor-mediated uptake of acetyl-LDL (21), arterial wall cholesteryl-ester/protein complexes (22), β-VLDL (very low-density lipoprotein) (23), and formaldehyde-albumin (6).

To further examine the differences between the AGE-protein receptor and the acetyl-LDL receptor, reciprocal crosscompetition experiments were performed in which cellular uptake and degradation of radioiodinated acetyl-LDL was evaluated in the presence of increasing concentrations of either unlabeled AGE-BSA, unlabeled FFI-BSA (Fig. 4) or unlabeled FFI-LDL (not shown). It was not possible to evaluate AGE-LDL, since the length of incubation time required for AGE formation on proteins (6–8 wk) precluded preparations of nonadenatured AGE-LDL. Cellular uptake and degradation of radiolabeled acetyl-LDL was effectively inhibited by addition of unlabeled acetyl-LDL. In con-
FIGURE 3. Competitive inhibition of $^{125}$I-AGE-BSA (top) and $^{125}$I-FFI-BSA (bottom) uptake by mouse peritoneal macrophages in the presence of increasing concentrations of potential polyanionic competitors: polyglutamic acid (O), polycytidylic acid (□), heparin (●), polynosinic acid (▲), polyanadenylic acid (■), and fucoidin (●). Each well received 10 μg (150 nM) of labeled AGE-BSA, 10 μg (150 nM) labeled FFI-BSA, and the indicated amount of unlabeled competitor. After incubation for 4 h at 37°C, the amount of radioactivity in the cells was determined in triplicate. Values are expressed as percent of control. The 100% values were 2.2 μg/mg for $^{125}$I-AGE-BSA and 0.125 μg/mg for $^{125}$I-FFI-BSA uptake.

Discussion

The studies described in this report demonstrate that the AGE-protein receptor is distinct from the previously described receptor for acetyl-LDL, although the possibility that acetyl-LDL is internalized to some extent by the AGE-receptor, as well as by its own receptor, cannot be excluded.

Negatively charged acetyl-LDL interfered with binding and degradation of AGE-BSA and FFI-BSA. However, the specific pattern of AGE- and FFI-BSA inhibition by polyanionic compounds was distinctly different from that of acetyl-LDL (21). Furthermore, reciprocal competition experiments showed no inhibition of acetyl-LDL binding and degradation by either AGE-BSA, FFI-BSA, or FFI-LDL.

These findings are consistent with the hypothesis that scavenger receptors are structurally distinct but related entities that show varying degrees of sensitivity to polyanionic compounds. The incubation conditions used in these experiments were selected to accelerate the formation of advanced glycosylation endproducts over a practical experimental period. Since it is the integral of glucose concentration over time that determines the extent of advanced glycosylation endproduct accumulation in vivo, with concentrations of glucose found in plasma (10 mM), this process would occur over a much longer period. That AGE formation does occur in vivo has recently (25) been demonstrated in collagen from nondi-
abetic subjects, where an age-dependent linear increase in AGE accumulation was found over a period of years.

The macrophage scavenger receptor for glucose-modified proteins, the specificity of which is defined by these studies, may have a unique biological significance, since it is the first such receptor that recognizes a ligand known to form extensively in vivo (24, 25). All body proteins investigated to date have been shown to form Schiff base and Amadori product adducts with glucose, in proportion to time and plasma glucose concentration (14). Although accumulation of these early nonenzymatic glycosylation products does not continue beyond the several weeks necessary to reach chemical equilibrium (26, 27), Amadori products on long-lived proteins such as collagen give rise over long periods of time to advanced glycosylation endproducts (AGE), which are recognized by the macrophage receptor described in this report (28). Via the AGE-protein receptor, macrophages would selectively recognize a time-dependent signal of protein modification, and thus degrade only senescent macromolecules. The AGE-protein receptor could, by this mechanism, have an important role in the regulation of extracellular protein turnover.

In vivo, the efficiency of this removal system is not complete, however, since advanced glycosylation products continue to accumulate over an individual's
entire lifespan as a function of both chronologic age and plasma glucose (14, 24). These accumulating AGE form intra- and intermolecular crosslinks (25, 29-31), which lead to the reduced elasticity of arteries, heart, and lungs associated with normal aging (31). Hyperglycemia-induced acceleration of this process may explain the premature degeneration of several tissues that is characteristically seen in long-term diabetes mellitus (32). In both normal aging and in diabetes, the rate at which aging-associated tissue changes take place may be profoundly affected by genetic and/or environmental factors that alter the efficiency of this receptor-mediated removal process. Increased future understanding of AGE receptor-mediated endocytosis should provide new insight into the common mechanisms underlying normal and diabetes accelerated aging.

Summary

A high-affinity macrophage receptor has been identified that recognizes proteins modified by a common in vivo process, long-term nonenzymatic reaction of glucose with proteins (AGE proteins). This receptor for glucose-modified proteins is now shown to be distinct from previously described scavenger receptors, using competition and crosscompetition experiments between AGE-modified protein and a variety of in vitro-modified scavenger receptor ligands, including unmodified BSA, unmodified low-density lipoproteins (LDL), acetyl-LDL, maleyl-BSA, and formaldehyde-treated BSA. Furthermore, the specific pattern of AGE-protein receptor inhibition by the polyanionic compounds polyinosinic acid, polyadenylic acid, polyglutamic acid, polycytidylic acid, fucoidin, and heparin was distinctly different from that of acetyl-LDL.

By thus selectively recognizing a time-dependent in vivo protein modification, macrophages may preferentially degrade senescent macromolecules, thereby having an important role in the regulation of extracellular protein turnover.

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