Advanced glycation end products are eliminated by scavenger-receptor-mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells

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Long-term incubation of proteins with glucose leads to the formation of advanced glycation end products (AGE). Physiological aspects of the catabolism of non-enzymically glycated proteins were studied *in vivo* and *in vitro*. AGE-modified BSA (AGE-BSA) was a mixture of high-M_r (cross-linked), monomeric and low-M_r (fragmented) AGE-BSA. After intravenous administration in rat, all three fractions of AGE-BSA accumulated extremely rapidly and almost exclusively in liver. Uptake in liver endothelial, Kupffer and parenchymal cells accounted for approx. 60%, 25% and 10–15% respectively of hepatic elimination. Both cross-linked and monomeric AGE-BSA were efficiently taken up and degraded in cultures of purified liver endothelial and Kupffer cells. Endocytosis of AGE-BSA by these cells was inhibited by several ligands for the scavenger receptor. Although ^{125}I-Hb was not endocytosed *in vitro*, ^{125}I-AGE-Hb was effectively endocytosed by a mechanism that was subject to inhibition by AGE-BSA. Endocytosis of N-terminal propeptide of type I procollagen, a physiological ligand for the scavenger receptor, was effectively inhibited by AGE-Hb and AGE-BSA. We conclude that AGE-modification renders macromolecules susceptible for elimination via the scavenger receptor of both liver endothelial and Kupffer cells.

INTRODUCTION

Long-term incubation of proteins with glucose leads, through the formation of early products such as Schiff base and Amadori products, to advanced glycation end products (AGE) [1]. The recent immunological demonstration of AGE-modified proteins in several animal and human tissues suggests a potential role of AGE-modification in the normal aging process [2] as well as in the pathogenesis of several diseases such as diabetic complications [3], atherosclerosis [4,5], haemodialysis-related amyloidosis [6] and Alzheimer's disease [7]. AGE-modified proteins are characterized physicochemically by a fluorescent brown colour and intramolecular and intermolecular cross-linking, and biologically by the specific recognition by the AGE-receptors [8–12]. Although it is generally accepted that macrophages are able to internalize AGE-proteins, conflicting results have been reported regarding the specificity of uptake. Vlassara et al. [13,14] reported on a unique novel macrophage receptor that recognized a possible AGE adduct, whereas Takata et al. [9] showed that the macrophage scavenger receptor was responsible for the endocytosis of AGE-protein. The latter group also showed that intravenously injected AGE-protein was rapidly cleared from the circulation by scavenger-receptor-mediated endocytosis in hepatic sinusoidal cells, although one study emphasized the non-specific binding of AGE to macrophages [15]. To understand the precise mechanism of elimination of AGE from the circulation, we set out to study the role of the two foremost populations of reticuloendothelial cells in the body, namely the Kupffer cells (KCs) and the liver endothelial cells (LECs). It has been well documented that these two cell types play pivotal roles in the removal of both non-physiological and physiological ligands for the scavenger receptor [16,17]. Although some macrophage scavenger receptors have been sequenced [18–20], the scavenger receptor of LECs has yet to be isolated and sequenced. In the present study we show that AGE-proteins are eliminated very efficiently from the circulation by scavenger-receptor-mediated endocytosis in both LECs and KCs.

EXPERIMENTAL

Ligands

AGE-BSA and AGE-human Hb were prepared as described previously [9,21]. Briefly, 2.0 g of BSA or 0.5 g of human Hb was dissolved in 10 ml of 0.5 M sodium phosphate buffer (pH 7.4) with 3.0 g of D-glucose. Each sample was sterilized by ultrafiltration, incubated at 37 °C for 40 weeks and dialysed against 20 mM sodium phosphate buffer (pH 7.4). The resulting AGE-proteins showed typical absorption and fluorescent spectra patterns that were indistinguishable from those reported previously [22], as well as a significant immunoreactivity to the anti-AGE antibody [23]. Determination by trinitrobenzenesulphonic acid methods showed that 50 out of 59 lysine residues were modified in AGE-BSA used in the present study. To prepare N-terminal propeptide of type I procollagen (PINP), type I procollagen was first purified from the culture medium of human osteosarcoma MG-63 cell line (American Type Culture Collection, Rockville, MD, U.S.A.) by precipitation with $(NH_4)_2SO_4$ and gel filtration on Sephacryl S-500 (Pharmacia, Uppsala, Sweden). The procollagen was digested with purified bacterial collagenase (grade CLSPA; Worthington). PINP was purified from the digest by anion-exchange HPLC (column Protein-Pak DEAE-5PW; Waters, Milford, MA, U.S.A.), gel filtration on

Abbreviations used: AcLDL, acetylated low-density lipoprotein; AGE, advanced glycation end products; FSA, formaldehyde-treated bovine serum albumin; KC, Kupffer cell; LEC, liver endothelial cell; MSR, macrophage scavenger receptor; PC, parenchymal cell; PINP and PIIINP, N-terminal propeptides of type I and type III procollagen; TC, tyramine cellobiose.

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Sephacryl S-300 (Pharmacia), and reverse-phase HPLC (Vydac, Hesperia, CA, U.S.A.). SDS/PAGE revealed a major band of M_r 14000, representing the N-terminal segment of the proα1(I) chain, and a minor band of M_r 7000, representing the N-terminal segment of the proα2(I) chain. The M_r values were obtained by comparison with collagenous standards. Heparin (free glycosaminoglycan chains) was a gift from Professor U. Lindahl (Uppsala, Sweden). Formaldehyde-treated BSA (FSA) and acetylated low-density lipoprotein (AcLDL) were prepared exactly as described [24–26]. Poly(I) was purchased from Sigma (St Louis, MO, U.S.A.); ^{125}I was from Amersham (Little Chalfont, Bucks., U.K.).

Labelling techniques

Proteins were labelled with ^{125}I (carrier-free Na^{125}I; Amersham) either by a direct reaction employing Iodobeads from Pierce Chemical Co. (Rockford, IL, U.S.A.) as described [27] or by conjugating the protein with ^{125}I-labelled tyraminyl cellobiose (TC) as described [28]. Low-density lipoprotein (LDL) and AcLDL were labelled only with the latter method. Either method gave specific radioactivities of (1–3) $\times$ 10^6 c.p.m. per μg of protein.

Determination of anatomical distribution and serum half-life ($t_{\frac{1}{2}}$)

The serum $t_{\frac{1}{2}}$ and the organ and hepatocellular distribution of intravenously administered labelled AGE-BSA were determined in rats as described [29]. ^{125}I-TC-labelled AGE-BSA (5 μg) was injected into a lateral tail vein. Blood sampling was started after 1 min by collecting 25 μl volumes from the tip of the tail into calibrated capillary tubes. The α- and β- phases of the elimination from blood were determined as described [29].

Isolation and culture of liver cells

The preparation of pure cultures of functionally intact KCs, LECs and parenchymal cells (PCs) from a single rat liver has been detailed elsewhere [30]. After collagenase perfusion of the liver, and isopycnic centrifugation of the resulting dispersed cells through Percoll (Pharmacia), pure monolayer cultures of KCs and LECs were established by selective attachment to substrates of fibronectin and glutaraldehyde-treated serum albumin respectively [31]. PCs were cleared of sinusoidal cells by sedimentation through Percoll as described [30].

Endocytosis studies *in vitro*

LEC cultures, established in 2 cm^2 wells and maintained in serum-free RPMI 1640 medium, were washed and supplied with fresh medium containing 1 % (w/v) serum albumin and labelled proteins. Incubations, performed for various lengths of time at 37 °C to measure endocytosis, were terminated by transferring the medium, along with one wash, to tubes containing 20 % (w/v) trichloroacetic acid. This precipitates only intact, undegraded protein or intermediate degradation products of high M_r. The extent of degradation was determined by measuring the radioactivities in pellet and supernatant after centrifugation. Cell-associated ligand was quantified by measuring the amount of label solubilized in PBS containing 1 % (w/v) SDS.

RESULTS

To study whether the modification of BSA by AGE generated cross-linked products, the M_r distribution of AGE-BSA was determined before and after conjugation with ^{125}I-TC by elution on a column of Sephacryl S-300 (Figure 1). Unlabelled protein exhibited two main peaks, one high-M_r peak probably representing cross-linked AGE-BSA (peak I), and a peak eluting at the position of monomeric BSA (peak II), taken to represent monomeric AGE-BSA. Fractionation of ^{125}I-TC-AGE-BSA revealed the presence of both peak I and peak II material, and an additional low-M_r peak (peak III), probably representing degraded material that could not be detected by absorbance at 280 nm. Peaks I, II and III were pooled as indicated in Figure 1 and were subsequently used as ligands to study the catabolic fate of AGE-BSA.

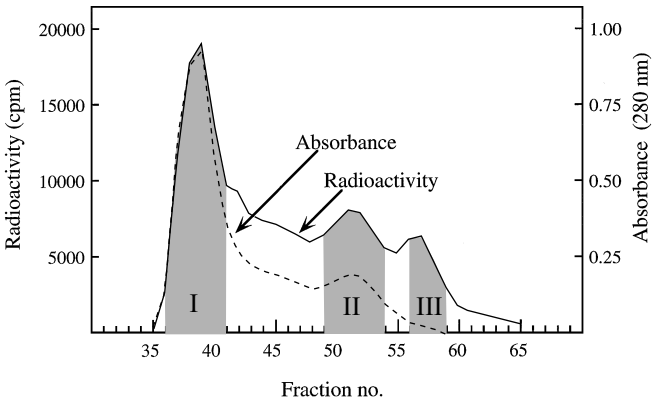

Figure 1 Gel chromatography of AGE-BSA

AGE-BSA (5 mg; broken line) or ^{125}I-TC-AGE-BSA [10^6 c.p.m. (approx. 1 μg); solid line] were applied to a column of Sephacryl S-300 (2.6 cm $\times$ 100 cm), and eluted with 0.2 M NH_4HCO_3 at a flow rate of 8 ml/h. Fractions of 2 ml were collected and analysed for protein (A_{280}) or radioactivity. Peaks I, II and III were pooled as indicated (shaded areas), freeze-dried and reconstituted in PBS. Peak I represents the exclusion volume, indicating that material in this peak is cross-linked; peak II co-eluted with monomeric BSA. The total volume was eluted at about fraction 70.

After intravenous administration, unfractionated ^{125}I-TC-AGE-BSA was removed from the circulation very rapidly (Figure 2): 70 % of the injected material was eliminated by a short $t_{\frac{1}{2}}$ (α) of 0.7 min, whereas the remaining 30 % was removed by a longer $t_{\frac{1}{2}}$ (β) of 5.6 min. Similar pharmacokinetic parameters were obtained when peak I or peak II ^{125}I-TC-AGE-BSA were injected (Table 1). More than 90 % of recovered radioactivity was found in liver when rats were killed 30 min after injection of unfractionated peak I or peak II ^{125}I-TC-AGE-BSA (Table 2).

The hepatocellular distribution of administered ^{125}I-TC-AGE-BSA was studied. Isolation of KCs, LECs and PCs after injection of ligand showed that unfractionated as well as peak I and peak II material distributed almost exclusively in KCs and LECs (Table 3). Uptake per PC was negligible. Because uptake per cell was the same in KC and LEC, and rat liver contains about 2.5 times more LECs than KCs [30], it could be calculated that the population of LECs was responsible for the elimination of more than 60 % of injected AGE-BSA, whereas the populations of KCs and PCs removed 25–30 % and 10–15 % respectively.

Next the kinetics and specificity of endocytosis of AGE-BSA were studied in purified cultures of isolated LECs or KCs. Continuous incubation of cultured LECs with a trace concentration (20 ng/ml) of unfractionated directly labelled ^{125}I-AGE-BSA showed avid uptake and degradation of the ligand (Figure 3). More than 30 % of added ligand was endocytosed after 1 h of incubation, and degradation proceeded linearly with time, more than 30 % being degraded after 3 h.

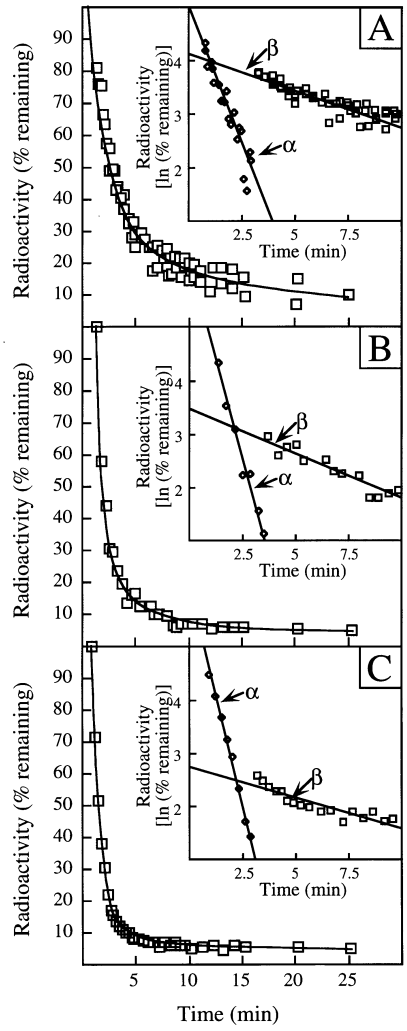

Figure 2 Clearance of AGE-BSA

Unfractionated ^{125}I-TC-AGE-BSA (5 μg, $n = 3$) (**A**), or peak I (**B**) ($n = 1$) or peak II (**C**) ($n = 1$) ^{125}I-TC-AGE-BSA was injected through the tail vein of rats, and radioactivity in blood samples was plotted against time after injection. Radioactivity at 1 min after injection was taken as 100%. Semilogarithmic plots (insets, □) were used to determine the slope, β, of the terminal phase ('β-phase'), taken from approx. 3 to 10 min after injection. The extrapolated values of the curve with slope β were subtracted from the experimental values obtained during the initial phase (1–3 min after injection) to give the curve with slope α (insets, ◇) describing the kinetics of the initial clearance phase ('α-phase'). See Table 1 for pharmacokinetic data calculated on the basis of the α and β slopes.

Because earlier studies had shown that AGE-BSA is endocytosed via the scavenger receptor in macrophages [9], we tested whether selected ligands for this receptor could compete with peak I and II ^{125}I-TC-AGE-BSA for uptake in KCs or LECs. To this end, purified cultures of KCs and LECs were incubated with trace amounts (20 ng/ml) of labelled fractions of AGE-BSA in the presence of excess amounts (100 μg/ml) of FSA, heparin or poly(I), which are all ligands for the scavenger receptor. Results presented in Figure 4 show that FSA and poly(I) inhibited endocytosis by approx. 70%, whereas the inhibition by heparin was approx. 25%. Endocytosis of directly labelled ^{125}I-AGE-BSA (peaks I, II and III) in the two cell types was similarly inhibited by ligands for the scavenger receptor (Figure 5). In particular, peak II material was inhibited significantly more avidly than peak I and III material, and the uptake of peak I ^{125}I-AGE-BSA, compared with peak I and II ^{125}I-TC-AGE-BSA and peak II and III ^{125}I-AGE-BSA, was far less affected by the presence of FSA and poly(I). Heparin and AcLDL were generally much weaker inhibitors than FSA, poly(I) and unlabelled unfractionated AGE-BSA (Figures 4 and 5). In general, the inhibitory power of all inhibiting ligands increased with decreasing molecular size of the labelled AGE-BSA.

To study whether other proteins could become ligands for the scavenger receptor after AGE modification, the endocytosis of ^{125}I-AGE-Hb and ^{125}I-Hb was determined. Results presented in Figure 6 show that labelled AGE-Hb, but not native Hb, was endocytosed in cultured LECs by a mechanism that could be inhibited by either unlabelled AGE-Hb or AGE-BSA.

N-terminal propeptides of types I and III procollagen (PINP and PIIINP) have been shown to be important physiological ligands for the scavenger receptor of LECs [17]. With this in mind we wanted to study whether endocytosis of labelled PINP could be inhibited by the presence of AGE-proteins. Indeed, both AGE-BSA and AGE-Hb effectively inhibited the endocytosis of labelled PINP (Figure 7).

Table 2 Anatomical distribution of intravenously injected ^{125}I-TC-AGE-BSA

The animals used in the serum $t_{\frac{1}{2}}$ studies (Figure 2) were analysed for anatomical distribution of radioactivity 60 min after injection. Approx. 80% of injected dose was recovered in the tissues listed in all experiments. The number of experiments is indicated by n; results for unfractionated ^{125}I-TC-AGE-BSA are means ± S.D.

	Uptake (% of total recovered)		
Source	Unfractionated ($n = 3$)	Peak I ($n = 1$)	Peak II ($n = 1$)
Liver	93.7 ± 2.1	91.0	93.9
Kidneys	2.4 ± 2.0	2.4	1.1
Urine	0.2 ± 0.1	0	0
Spleen	3.5 ± 0.5	3.5	3.7
Lungs	0.1 ± < 0.1	0.2	0.1
Heart	< 0.1 ± < 0.1	< 0.1	< 0.1
Thymus	< 0.1 ± < 0.1	< 0.1	< 0.1
Brain	< 0.1 ± < 0.1	< 0.1	< 0.1
Blood	2.3 ± 1.1	2.4	1.0

Table 1 Pharmacokinetic parameters of elimination of ^{125}I-TC-AGE-BSA from the circulation

The number of experiments is indicated by n; results for unfractionated ^{125}I-TC-AGE-BSA are means ± S.D.

Preparation of ^{125}I-TC-AGE-BSA	$t_{\frac{1}{2}}$ (α) (min)	Clearance with $t_{\frac{1}{2}}$ (α) (%)	$t_{\frac{1}{2}}$ (β) (min)	Clearance with $t_{\frac{1}{2}}$(β) (%)
Unfractionated ($n = 3$)	0.7 ± 0.1	70.2 ± 7.7	5.6 ± 0.9	29.8 ± 7.7
Peak I ($n = 1$)	0.5	94.1	4.1	5.9
Peak II ($n = 1$)	0.4	96.7	6.0	3.31

Table 3 Hepatocellular distribution of intravenously injected ^{125}I-TC-AGE-BSA

The animals used in the serum $t_{\frac{1}{2}}$ studies (Figure 2 and Table 2) were analysed for radioactivity in purified liver cells 60 min after injection. Radioactivity per cell is normalized to uptake per Kupffer cell. Uptake per cell population is the relative uptake per total cell population in the intact liver (calculated from the relationship that KC, LEC and PC occur in rat liver in the ratio 1:2.5:7.7 [30]). The number of experiments is indicated by *n*.

Preparation of ^{125}I-TC-AGE-BSA	Kupffer cells		Liver endothelial cells		Parenchymal cells	
	Uptake per cell	Uptake per cell population	Uptake per cell	Uptake per cell population	Uptake per cell	Uptake per cell population
Unfractionated ($n = 3$)	1.00	24.34	1.12	60.13	0.09	15.53
Peak I ($n = 1$)	1.00	28.04	0.89	62.55	0.04	9.39
Peak II ($n = 1$)	1.00	23.95	1.11	66.29	0.05	9.76

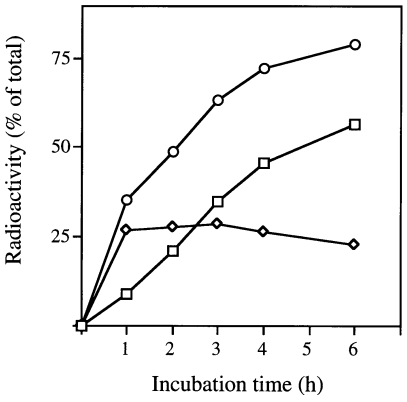

Figure 3 Kinetics of endocytosis of ^{125}I-AGE-BSA in cultured LECs

^{125}I-AGE-BSA (10 ng) was incubated with cultures of LECs in 2 cm^2 dishes, and cell-associated (◇) and degraded (□) ligand were determined after various periods as described in the Experimental section. The extent of endocytosis (○) is the sum of cell-associated (◇) and degraded (□) ligand.

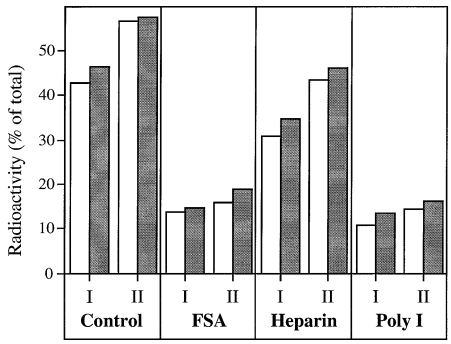

Figure 4 Specificity of endocytosis of ^{125}I-TC-AGE-BSA in purified cultures of hepatic sinusoidal cells

Cultures of KCs (open bars) or LECs (shaded bars) were incubated with 10 ng of high-M_r peak I (I) or monomeric peak II (II) material from the fractionation of ^{125}I-TC-AGE-BSA on Sephacryl S-300 (see Figure 1). Incubation was performed for 2 h at 37 °C in 2 cm^2 dishes in the absence (control) or presence of 100 μg/ml FSA, heparin or poly(I). Results represent percentages of total added ligand.

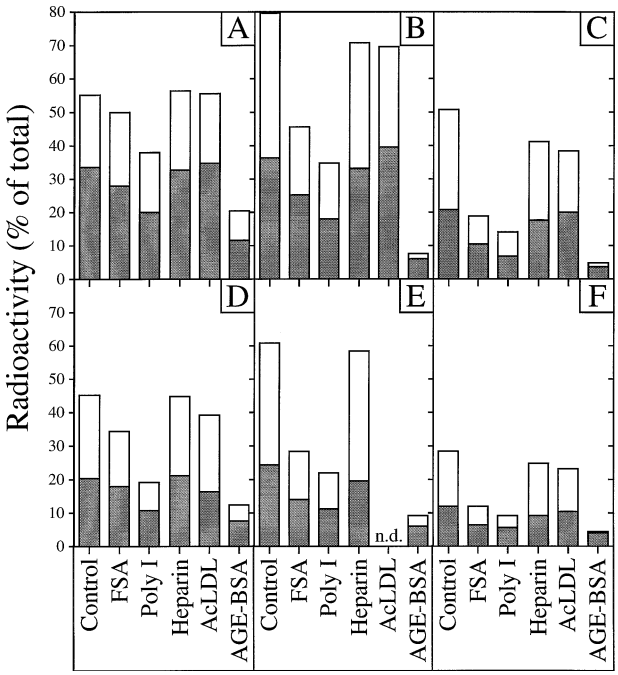

Figure 5 Specificity of endocytosis of ^{125}I-AGE-BSA in purified cultures of hepatic sinusoidal cells

Cultures of LECs (**A**, **B**, **C**) or KCs (**D**, **E**, **F**) were incubated with 10 ng of high-M_r peak I (**A**, **D**), monomeric peak II (**B**, **E**) or low-M_r peak III (**C**, **F**) material from the fractionation of ^{125}I-AGE-BSA on Sephacryl S-300 (see Figure 1). Incubation was performed for 2 h at 37 °C in 2 cm^2 dishes in the absence (control) or presence of 100 μg/ml FSA, poly(I), heparin, AcLDL or AGE-BSA. The extent of endocytosis is the sum of cell-associated (shaded part of bars) and degraded (open part of bars) ligand. Results represent percentages of total added ligand. Abbreviation: n.d., not done.

DISCUSSION

In line with Takata et al. [9], we found that AGE-proteins are efficiently cleared from the circulation by scavenger-receptor-mediated endocytosis in hepatic sinusoidal cells. By labelling AGE-BSA with ^{125}I-TC, which is trapped intralysosomally after the carrier protein has been endocytosed and degraded, we showed that intravenously administered AGE-BSA is taken up to the same extent per KC and LEC. This finding was surprising in that soluble macromolecular ligands that are cleared from the circulation by scavenger-receptor-mediated endocytosis are normally taken up almost exclusively by LECs [16,17]. An exception to this rule is the elimination of circulating oxidized LDL, which is taken up by both LECs and KCs [32].

The reactions leading to the formation of AGE might involve extensive cross-linking of proteins [33]. Consequently we analysed the preparation of AGE-BSA for the presence of high-M_r products on gel chromatography, and found that it contained

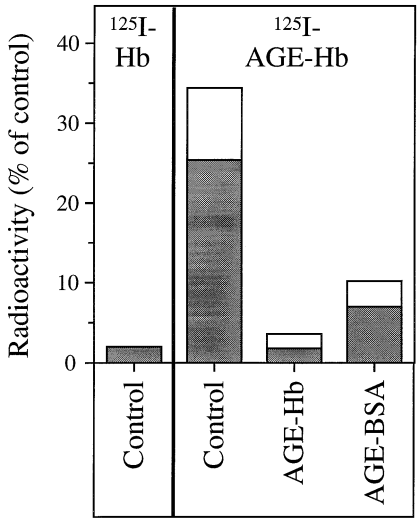

Figure 6 Specificity of endocytosis of ^{125}I-AGE-Hb in purified cultures of LECs

Cultures of LECs were incubated for 1 h at 37 °C in 2 cm^2 dishes with 10 ng of ^{125}I-AGE-Hb in the absence (control) or presence of 100 μg/ml AGE-Hb or AGE-BSA. The result from incubation with 10 ng of ^{125}I-Hb alone is included to indicate that native Hb is recognized only to a very small extent by LECs. The extent of endocytosis is the sum of cell-associated (shaded part of bars) and degraded (open part of bars) ligand. Results represent percentages of total added ligand.

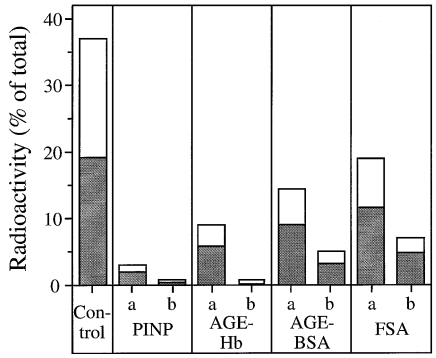

Figure 7 Specificity of endocytosis of ^{125}I-PINP in purified cultures of LECs

Cultures of LECs were incubated for 1 h at 37 °C in 2 cm^2 dishes with 10 ng of ^{125}I-PINP in the absence (control) or presence of AGE-Hb, AGE-BSA or FSA. The concentrations of the competitive ligands were either 10 μg/ml (**a**) or 100 μg/ml (**b**). The extent of endocytosis is the sum of cell-associated (shaded area of bars) and degraded (open area of bars) ligand. Results represent percentages of total added ligand.

large amounts of high-M_r cross-linked material in addition to the monomeric material. To study whether the unexpectedly avid uptake in KCs was due to the presence of cross-linked or aggregated AGE-BSA, we injected fractions of cross-linked or monomeric ligand and determined the hepatocellular distribution. Interestingly, the extent of cross-linking did not seem to influence the targeting of AGE-BSA to KCs or LECs. This observation *in vivo*, along with the finding that the specificity of endocytosis of AGE-BSA in purified cultures of LECs and KCs was indistinguishable, suggests that the scavenger receptors in KCs and LECs responsible for the uptake of this modified protein share the same ligand specificity properties.

Uptake of TC-labelled peak I and II AGE-BSA, directly labelled peak II and III AGE-BSA, and unfractionated directly labelled AGE-BSA, was inhibited by 50–70 % by FSA. It was therefore unexpected to find that endocytosis of directly labelled aggregated (peak I) AGE-BSA was only marginally inhibited by both FSA and poly(I). We are at present unable to explain this different behaviour of peak I directly labelled AGE-BSA.

The term scavenger receptor was originally coined to denote a receptor species in macrophages that recognizes and endocytoses modified proteins, e.g. AcLDL and malondialdehyde-treated serum albumin [34]. It was soon found that this receptor recognizes an array of ligands with only one property in common: a high net negative charge. These early studies gave no clue as to the physiological relevance of this receptor. Later, oxidized LDL was found to be a ligand for the scavenger receptor. Although the existence of this modified lipoprotein *in vivo* is less controversial than AcLDL or any of the other test ligands for the scavenger receptor, a clear physiological function of this receptor was found by Melkko et al. [17], who showed that both PINP and PIIINP are avidly cleared from the circulation by the scavenger receptor in LECs. It is highly likely that an increased number of physiological ligands for this receptor will be discovered in the future. The present work has established that both LECs and KCs carry scavenger receptor(s) that recognize AGE-proteins. Several studies have suggested that such ligands are present in normal individuals, and in increased amounts in diabetics [35,36].

The correlation of the extent of AGE-modification with cellular recognition of AGE-proteins via the scavenger receptors is important and is essential for understanding the physiological role(s) of the scavenger receptors in the elimination of these waste molecules. The protein modification *in vivo* is expected to be much milder than that occurring *in vitro*. It is not clear therefore whether proteins *in vivo* are subjected to AGE-modification to such an extent that it will be recognized by the receptor. In this connection, recent studies suggested that carboxymethyl-lysine is a major AGE structure in AGE-proteins [37,38]. However, amino acid analyses of AGE-BSA used in the present study showed that 10 out of 59 lysine residues of BSA were modified by carboxymethylation and 40 residues were modified in other ways. Therefore it is possible that different types of chemical modification occur to a protein in a synergistic way *in situ*. On a related issue, it is also important to determine the structure(s) of AGE-proteins recognized by cells. A previous study demonstrated that AGE-BSA is recognized like AcLDL by macrophages or macrophage-derived cells through the macrophage scavenger receptor. This receptor is expected to recognize a structure(s) that AGE-proteins and AcLDL have in common. Ligands for the macrophage scavenger receptor include AcLDL, oxidized LDL and several polyanions (polyvinyl sulphate, dextran sulphate, fucoidin, polyguanosinic acid and polyinosinic acid). In contrast, ligands for the AGE receptor include AGE-proteins and aldehyde-modified proteins [9,39] as well as the polyanions described above for the macrophage scavenger receptor. It is clearly difficult to identify a haptenic structure common to AGE-proteins and AcLDL. However, both AGE-proteins and AcLDL themselves are polyanions in nature, and both receptors showed a very similar polyanion specificity. Therefore it is likely that the polyanionic nature of AGE-proteins (as a cluster of polyanionic charges), rather than a common AGE structure, plays a major role as a recognition signal for AGE-proteins. Further studies are needed to resolve this issue.

Shaw and Crabbe [15] emphasized the non-specific binding of

AGE to macrophages. Their data were obtained from binding experiments with cell suspension systems, whereas our results were obtained with cells that adhered tightly to the growth substrate in culture plates. From our experience, binding of ^{125}I-AGE-BSA to the murine macrophage RAW cell line or peritoneal macrophages in cell suspension was significantly (5–10 times) higher than binding observed with adherent cell culture systems. Further, a large proportion of ^{125}I-AGE-BSA bound to these cells in suspension corresponded to non-specific binding. However, the cellular binding of ^{125}I-AGE-BSA in the adherent cell culture system showed a specific binding with a typical dose-dependent saturation curve [9,40,41]. Therefore the marked difference of the present results from those reported by Shaw and Crabbe might lie in the experimental systems.

Although two very similar macrophage scavenger receptors (MSR I and II) have been cloned and sequenced [18,19], several lines of evidence indicate the existence of other types of receptor with very similar ligand specificities. AGE-BSA was effectively endocytosed by Chinese hamster ovary (CHO) cells over-expressing bovine MSR II, whereas wild-type CHO cells did not show such an effect, suggesting a major role of MSR in endocytic uptake of AGE-proteins by macrophages or macrophage-derived cells [40]. Moreover, a recent study with peritoneal macrophages obtained from homozygous MSR-knockout mice showed that the endocytic capacity of these cells for AcLDL, oxidized LDL and AGE-BSA was decreased to 22%, 34% and 33% respectively of wild-type macrophages [41]. Thus approx. two-thirds of the endocytic uptake of AGE-proteins by the mouse macrophages is mediated by MSR, whereas the remaining one-third is mediated by other macrophage receptors such as FcγRII-B2 [42], CD36 [43], SR-BI [44], RAGE [45] and MARCO [20]. In addition, the presence of other types of scavenger receptor in macrophages and macrophage-related cells has also been suggested. Ottnad et al. [46] and Sambrano and Steinberg [47] demonstrated that in mouse peritoneal macrophages a 94000–97000-M_r oxidized-LDL-binding protein also recognized oxidatively damaged cells and apoptotic cells. Sparrow et al. [48] and Arai et al. [49] proposed the presence of a scavenger receptor specific for oxidized LDL but not for AcLDL in mouse peritoneal macrophages. Van Berkel and co-workers [32,50] identified a 95000-M_r protein as a putative oxidized LDL receptor in rat KCs. Melkko et al. [17] proposed that the endocytic uptake of PINP and PIIINP by rat LECs is mediated by a receptor that is partly competed for by AcLDL. Taken together, these results suggest the presence of multiple scavenger receptors that recognize atherogenic molecules such as modified LDL and AGE-proteins. However, further studies are needed to determine the exact contribution of each receptor *in vivo* to the endocytic uptake of these proteins by LECs, macrophages or macrophage-derived foam cells present in atherosclerotic lesions.

It can be hypothesized that atherosclerosis might develop as an indirect consequence of a disability of the hepatic scavenger receptor to eliminate atherogenic substances from the circulation. If the generation of ligands for the scavenger receptors of LECs and KCs exceeds the clearance capacity of these cells, or if their endocytic activities are modulated, atherogenic molecules might escape hepatic sequestration and reach cells of the intima of larger extrahepatic vessels, causing the development of atherosclerosis. This idea is not far-fetched because recent studies show that endocytic activity in LECs might be significantly upregulated by interleukin-1β or tumour necrosis factor α [51], or down-regulated by nitric oxide [52]. Thus the most important site of clearance of atherogenic molecules from the circulation seems to be under the control of inflammatory mediators.

The presence *in vivo* of AGE-modified plasma proteins and peptides [53,54] and AGE-modified LDL [55] has been reported. This fact, along with our present findings, strongly suggests that scavenger-receptor-mediated elimination of these waste molecules in LECs and KCs represents a major physiological mechanism preventing pathogenesis of several diseases such as diabetic complications, atherosclerosis, haemodialysis-related amyloidosis and Alzheimer's disease.

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