

Scavenger Receptors on Sinusoidal Liver Endothelial Cells Are Involved in the Uptake of Aldehyde-Modified Proteins

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ABSTRACT

Scavenger receptors on sinusoidal liver endothelial cells (SECs) eliminate potentially harmful modified proteins circulating through the liver. It was shown recently that aldehyde-modified proteins bind to scavenger receptors and are associated with the development/progression of alcoholic liver diseases. For these studies, rat livers were perfused in situ with ¹²⁵I-formaldehyde-bovine serum albumin (f-Alb) or ¹²⁵I-malondialdehyde-acetaldehyde-bovine serum albumin (MAA-Alb) in the presence of known scavenger receptor ligands as inhibitors. Reverse transcription-polymerase chain reaction (RT-PCR) analysis and scavenger receptor Type A (SRA) knock-out mice were used to assess the role of these receptors in mediating immune responses. The degradation of ¹²⁵I-f-Alb or ¹²⁵I-MAA-Alb in

whole livers and isolated SECs can be inhibited by known scavenger receptor ligands, including f-Alb, maleylated bovine albumin, and fucoidan. ¹²⁵I-f-Alb could not be completely inhibited by MAA-Alb. In contrast, ¹²⁵I-MAA-Alb was only partially inhibited with advanced glycosylated endproduct albumin. RT-PCR data show the presence of a number of scavenger receptors on SECs that may be responsible for the binding of MAA-modified proteins. SRA seems to be one of these receptors involved in the effects mediated by MAA-modified proteins. In a study using SRA knockout mice, it was shown that a decreased antibody response to MAA-Alb resulted. By RT-PCR, CD36, LOX-1, and SR-AI are the scavenger receptors most likely involved in the degradation of MAA-Alb.

Scavenger receptors were first described in macrophage as alternative receptors for the low-density lipoprotein receptor that is responsible for the uptake of excessive cholesterol, leading to the formation of foam cells (Terpstra et al., 2000). Since this original description, a broad array of ligands for several classes of scavenger receptors have been described previously (Terpstra et al., 2000). In general, organic acid anhydrides, peroxides, and aldehydes (Brown et al., 1980) result in the modification of positively charged lysine resi-

dues to alter the charge on proteins to make them ideal scavenger receptor ligands (Alaiz et al., 1994). Receptors specifically recognizing aldehyde modified proteins were first described using formaldehyde-modified albumin (f-Alb) (Brown et al., 1980; Horiuchi et al., 1986; Takata et al., 1988, 1989; Steinbrecher et al., 1989). The specificity of this receptor was demonstrated by inhibiting the binding of f-Alb modified with a number of other aldehydes (glycolaldehyde, DL-glyceraldehyde, and propionaldehyde) (Horiuchi et al., 1986).

Scavenger receptors have been implicated in diabetes by binding altered self proteins (Uchida, 2000). The glycation of proteins is a complex series of reactions between reducing sugars and amino groups of proteins, resulting in many aldehydes and ketones being formed such as α -ketoaldehydes (glyoxal, 3-deoxyglucosone, and glucosone) (Uchida, 2000). The aldehyde methylglyoxal, a glycolytic product, is produced from the autooxidation of sugars and glycation (Uchida, 2000). These α -ketoaldehydes represent highly reactive intermedi-

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ABBREVIATIONS: f-Alb, formaldehyde-modified albumin; Alb, bovine serum albumin; AGE, advanced glycation end-products; Maley-Alb, maleylated bovine albumin; SEC, sinusoidal liver endothelial cell; MAA, malondialdehyde-acetaldehyde adduct; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); SRA, scavenger receptor A; KO, knockout; WT, wild type; TNF, tumor necrosis factor.

ates in the glycation process that results in the formation of advanced glycation end-products (AGE) (Uchida, 2000). The binding of AGE-modified Alb has been shown experimentally to be inhibited with Alb modified by formaldehyde and glycolaldehyde, indicating specificity to a scavenger receptor (Takata et al., 1988). This inhibition shows the broad range of binding that the scavenger receptor has to aldehyde modified proteins. Many scavenger receptors have been shown to recognize proteins modified with AGE including scavenger receptor class A types I and II (Araki et al., 1995; Suzuki et al., 1997), and scavenger receptor B (class I and CD36) (Ohgami et al., 2001a,b).

Oxidized low-density lipoprotein has been shown to be an important mediator of atherosclerosis and is formed by adduct formation between apolipoprotein and reactive aldehydes such as 4-hydroxynonenal and malondialdehyde (Palinski et al., 1990; Hoff and O'Neil, 1993). The mechanism by which these aldehyde-modified proteins become immunogenic has been reported to be mediated through the scavenger receptor class A (Nicoletti et al., 1999).

Proteins modified with maleic anhydride (Maley-Alb) have also been shown to cause their effects via a scavenger receptor (Abraham et al., 1995, 1997). These modified proteins have the ability to bind to scavenger receptors and induce antibody and T-cell responses against the native modified protein. In fact, Abraham et al. (1995) demonstrated the breaking of tolerance when mouse serum albumin was modified with maleic anhydride.

The finding that MAA-Alb is bound and degraded by sinusoidal liver endothelial cells (SECs) in a manner similar to that of acetaldehyde-Alb and f-Alb (Thiele et al., 1996, 1999; Duryee et al., 2003), presents the possibility that it enters the cell through a scavenger receptor. To begin determining whether MAA-Alb exerts its effects via a scavenger receptor, known ligands such as AGE-Alb, f-Alb, Maley-Alb, and fucoidan were used as competitors. Therefore, it was the purpose of these studies to determine whether MAA-Alb and f-Alb bind to SECs via scavenger receptors and whether alcohol impairs these inhibitions.

Materials and Methods

Rats. Male Wistar rats purchased from Charles River Laboratories (Wilmington, MA) were maintained on a Purina rat chow diet until they reached a weight of 140 to 150 g. As described previously, rats were fed the Lieber-DeCarli liquid diet from Dyets, Inc. (Bethlehem, PA) for 6 weeks (Lieber and DeCarli, 1982; Duryee et al., 2003). All animals were allowed free access to their food and/or water up to 1 h before sacrifice. All procedures were approved by the Animal Subcommittee of the Omaha VA Medical Center and are in accordance with the National Institutes of Health *Guidelines on the Care and Use of Laboratory Animals*.

Chemicals and Proteins. Bovine serum albumin (Alb) was purchased from Calbiochem (San Diego, CA). Acetaldehyde was obtained from Aldrich Chemical Co. (Milwaukee, WI). Malondialdehyde was obtained as the sodium salt by treatment of tetramethoxypropane (Aldrich Chemical Co.) with NaOH, according to the method of Kikugawa and Ido (1984). Trichloroacetic acid, phosphotungstic acid, formaldehyde, maleic anhydride, fucoidan, fetuin, and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium ^{125}I was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Preparation and Labeling of Ligands. Alb was labeled with ^{125}I by the chloramine-T method (^{125}I -Alb), with resulting specific

activities between 2800 and 3200 cpm/ng (McConahey and Dixon, 1980). Proteins were first labeled with ^{125}I , and then modified with formaldehyde or MAA. Modification of Alb with formaldehyde was done using the methods of Mego and Horiuchi (Mego et al., 1967; Horiuchi et al., 1986). Malondialdehyde and acetaldehyde were incubated with Alb to form the MAA-Alb adduct as described previously by Tuma et al. (1996). The maleylation of Alb was prepared by a reaction with maleic anhydride as directed by Barnes et al. (1988). The advanced glycosylation endproduct was prepared by incubation with 0.5 M glucose for 6 weeks as described previously (Vlassara et al., 1985; Yang et al., 1991). Protein concentrations were determined by the method of Lowry (1951). ^{125}I radioactivity was assessed using a Cobra II γ -spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA).

In Situ Isolated Liver Perfusion. After 6 weeks of the Lieber DeCarli liquid diet or regular chow diet, rats were anesthetized with isoflurane and perfused as described previously (Thiele et al., 1999; Duryee et al., 2003). In brief, the liver was equilibrated for 15 min with oxygenated Krebs-Ringer-bicarbonate buffer containing 1% Alb, followed by a 2 mg/200 ml solution of ^{125}I -modified Alb, MAA-Alb, or f-Alb that was recirculated for 3 h through the liver at a flow rate of 20 ml/min (2.5–3 ml/min/g of liver). The addition of 100 \times unlabeled MAA-Alb, f-Alb, Maley-Alb, AGE-Alb, fucoidan, or fetuin was mixed with the above radioactive ligand for inhibition studies. After 3 h of incubation, 0.5-ml aliquots were removed and 2.0 ml of 20% trichloroacetic acid and 2% phosphotungstic acid was added to precipitate intact proteins. Measuring the amount of acid-soluble radioactivity in the perfusate was used to assess ligand degradation.

Isolation of SECs. Sinusoidal liver endothelial cells were prepared by perfusion and differential centrifugation methods as described previously (Braet et al., 1994; Duryee et al., 2004). Cell number and viability was determined by trypan blue exclusion and counting on a hemocytometer.

Viability was routinely greater than 85%. SEC purity was determined by antibodies specific for the various cell types of the liver as assessed by a fluorescence-activated cell scanner (FACSCalibur; BD Biosciences, San Jose, CA) as reported previously (Duryee et al., 2004) and found to be greater than 90% pure.

In Vitro Degradation Inhibition Assays. Inhibition of ^{125}I -MAA-Alb or ^{125}I -f-Alb degradation by SECs was performed by using known scavenger receptor ligands such as f-Alb, Maley-Alb, AGE-Alb, and fucoidan. In general, cell suspensions (1×10^6 cells/ml in M199/Ham's F12 media containing 1% Alb) were incubated in 24-well tissue culture plates with the ligand only (25 $\mu\text{g}/\text{ml}$) or ligand plus inhibitor at 100 \times (2.5 mg) for 3 h. Samples of the supernatant were taken at 0 and 3 h and added to an ice-cold, 20% trichloroacetic acid/2% phosphotungstic acid solution for determination of acid soluble radioactivity (degradation). After 20 min on ice, the solution was centrifuged for 10 min at 350g, and radioactivity in the supernatant determined using a Cobra II gamma-counter. Inhibition of MAA-Alb or f-Alb was determined by the lack of radioactive uptake by the SECs of the labeled ligand.

In Situ Inhibition of Degradation Assay Using Alcohol-Fed Rats. Male Wistar rats were fed an alcohol diet for 6 weeks as described above. Animals were then subjected to exactly the same procedure as described for the in situ perfusion of chow-fed animals. The pair-fed and alcohol-fed animals were in situ-perfused with identical ligands and inhibitors.

RT-PCR for Scavenger Receptors. Chow-fed rats were subjected to surgery to isolate the mesenteric vein for injection. Using aseptic technique, the abdominal cavity was opened, and the stomach and intestines were placed on sterile gauze. The mesenteric vein was located and injected with 0.5 ml of PBS, 500 μg of Alb, or 500 μg of MAA-Alb. The mesenteric vein was allowed to clot, the bowel was replaced in the abdominal cavity, and the peritoneal cavity was closed with sutures and staples. Rats were watched until awake and allowed ad libitum access to food and water overnight. After 16 h the animals were sacrificed. Livers

were flushed with saline and removed, and SECs were isolated as outlined above. RNA from whole liver and SECs was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instructions. RNA was quantified and integrity-verified by electrophoresis of 1 μ g of RNA on a 1% agarose gel stained with ethidium bromide. RNA bands were detected by exposure on a Fluor-S multi-imager and analyzed by Quantity One software (Bio-Rad, Hercules, CA). RT-PCR was performed using a QIAGEN one-step RT-PCR reaction kit. In brief, 1 μ g of RNA was mixed with dNTP and enzyme supplied by Qiagen with each set of primers to a final concentration of 1 μ M. Table 1 shows the primer sequences used to determine the presence of scavenger receptors. Primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Amplifications were performed using an Eppendorf Mastercycler Personal (Brinkmann Instruments, Westbury, NY) as follows: denaturation (1 min, 94°C), annealing (1 min,

56°C), and extension (1 min, 72°C), which were carried out for 25 amplification cycles. PCR products were analyzed by electrophoresis in 1% agarose containing ethidium bromide. Fragment size was verified using a 100-bp DNA ladder standard (New England Biolabs, Beverly, MA) and gels exposed as described above.

Scavenger Receptor A Knockout Mice. Scavenger receptor A knockout (SRA-KO) and wild-type (SRA-WT) mice were a gift of Dr. Hiroshi Suzuki (Suzuki et al., 1997). In brief, mice were of an ICR background and maintained by mating brother to sister. These mice were screened for wild-type by incubating splenocytes with the fluorescent probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate and analyzing uptake with the FACSCalibur device as described previously (Nicoletti et al., 1999). SRA-KO and SRA-WT mice were injected with 25 μ g/ml of Alb, MAA-Alb, and a phosphate control. Mice were injected once a week for 6 weeks as described previously for the MAA-adduct injection (Thiele et al., 1998). At the

TABLE 1
Scavenger receptor primer sequences used for RT-PCR studies

5'-SR-AI (5'-ATGACAAAGAGATGACAGAGAAT-3')	3'-SR-AI (5'-TTATGAAGTACAAGTGACCCC-3')
5'-SR-BI (5'-ATGGGCGTCAGCTCCAG-3')	3'-SR-BI (5'-CTATAGCTTGGCCTTCTTGC-3')
5'-CD36 (5'-ATGGGCTGCGATCGGAAC-3')	3'-CD36 (5'-TTATTTTCCATCTCTTAGATCTGC-3')
5'-LOX1 (5'-ATGAATTGGAAATGGCTTTTG-3')	3'-LOX1 (5'-TCACTGAGTTAGCAATAAATTTG-3')
5'-CD14 (5'-ATGAAGCTTATGCTCGGCTTG-3')	3'-CD14 (5'-TTAAACAAGAGGCGATGTCC-3')
5'-TNF (5'-CCACGCTCTTCTGTCTACTG-3')	3'-TNF (5'-GGAGGCTAGCTTTCTCCTG-3')
5'-GAPDH (5'-ACGGAAGGCATGCCAGTGA-3')	3'-GAPDH (5'-ACATGTTCCAGTATGACTCT-3')

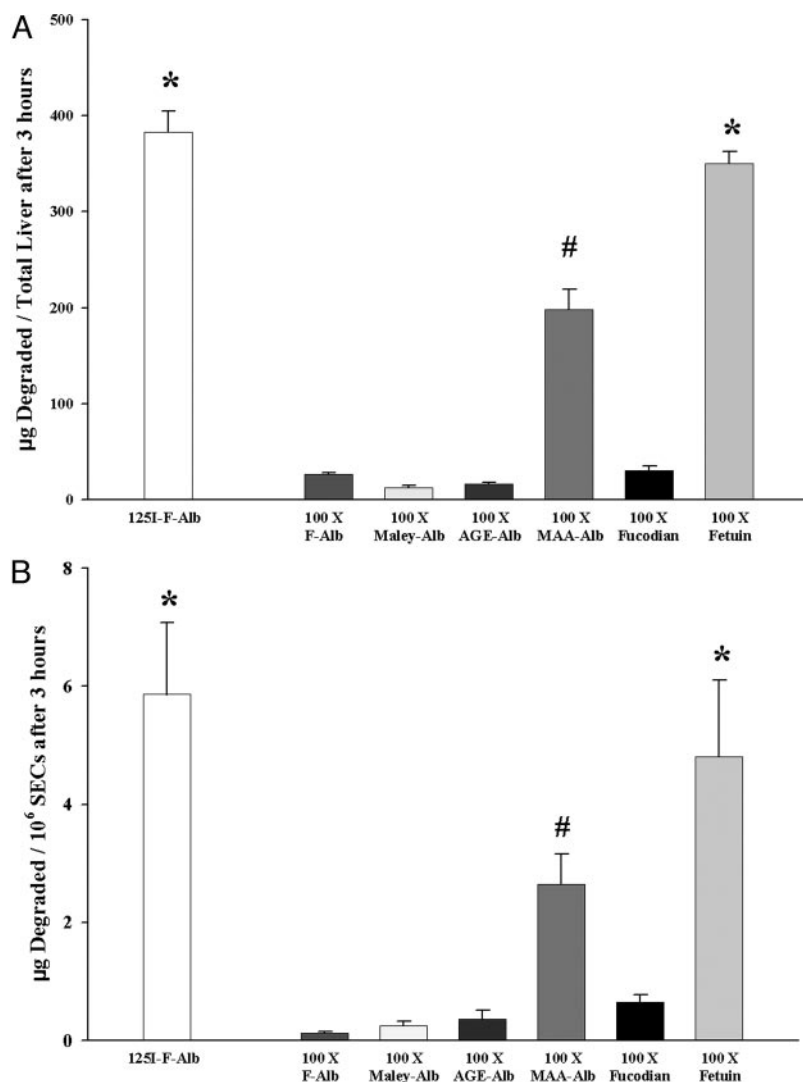


Fig. 1. Inhibition of 125 I-f-Alb degradation by in situ perfused livers (A) and isolated SECs (B) of chow-fed rats. Livers or isolated SECs were incubated for 3 h with 10 μ g/ml of 125 I-f-Alb and 100-fold concentrations of either unlabeled f-Alb, Maley-Alb, AGE-Alb, MAA-Alb, fucodan, or fetuin. Intact protein was precipitated and acid soluble radioactivity (protein degradation) in the supernatant was determined. Results are expressed as means \pm S.D. ($n = 6$ in each group). *, $P < 0.001$ indicates significant differences from the 125 I-f-Alb alone. #, $P < 0.01$ indicates a significant difference from 125 I-f-Alb.

end of this incubation period, the mice were bled, and serum was assayed for the presence of the MAA antibody by direct enzyme-linked immunosorbent assay.

Statistical Analysis. Results are expressed as means \pm S.E.M. Statistical significance was achieved if *P* values were less than 0.05. All statistical analysis was performed using analysis of variance in SigmaStat (SPSS Inc., Chicago, IL).

Results

Inhibition of ^{125}I -f-Alb Degradation. Previous studies have reported that f-Alb will enter SECs via scavenger receptors (Horiuchi et al., 1986; Thiele et al., 1998; Hansen et al., 2002a). To begin examining which scavenger receptors are responsible for degrading aldehyde modified proteins, ^{125}I -f-Alb degradation was inhibited with known ligands that bind scavenger receptors in an in situ liver perfusion model. In these studies, livers from chow-fed rats were perfused for 3 h with ^{125}I -f-Alb and inhibited with 100-fold concentrations of unlabeled f-Alb, Maley-Alb, AGE-Alb, MAA-Alb, fucoidan, and fetuin, a nonspecific polyanionic compound. As shown in Fig. 1A, normal degradation of ^{125}I -f-Alb (400 $\mu\text{g}/\text{ml}$) was completely inhibited with 100-fold f-Alb, Maley-Alb, AGE-Alb, and fucoidan. However, MAA-Alb inhibited the degra-

dation of ^{125}I -f-Alb degradation by only half. The nonspecific control, fetuin, resulted in no inhibition. Data from these studies demonstrate the effects of these ligands in whole liver and not a specific cell type. Therefore, SECs were isolated from chow-fed rats and subjected to similar studies in an in vitro model system. Degradation of ^{125}I -f-Alb in isolated SECs resulted in the degradation of 6 μg after a 3-h period (Fig. 1B). Incubation with known scavenger receptor ligands demonstrated complete inhibition of degradation when f-Alb, Maley-Alb, AGE-Alb, and fucoidan were used in these experiments. As shown in Fig. 1B, there was only a 50% reduction in degradation when MAA-Alb was used as the competing ligand. Fetuin, the negative control, had no effect on ^{125}I -f-Alb degradation.

Inhibition of ^{125}I -MAA-Alb Degradation. Previous studies performed in our laboratory have demonstrated that MAA-Alb is degraded by both SECs (Duryee et al., 2003) and peritoneal macrophage (Thiele et al., 1998), but the mechanism by which MAA-Alb enters the cell is relatively unknown. These studies were designed to investigate whether scavenger receptors were involved. Livers from chow-fed rats were perfused for 3 h with ^{125}I -MAA-Alb and inhibited with 100-fold concentrations of unlabeled MAA-Alb, Maley-Alb,

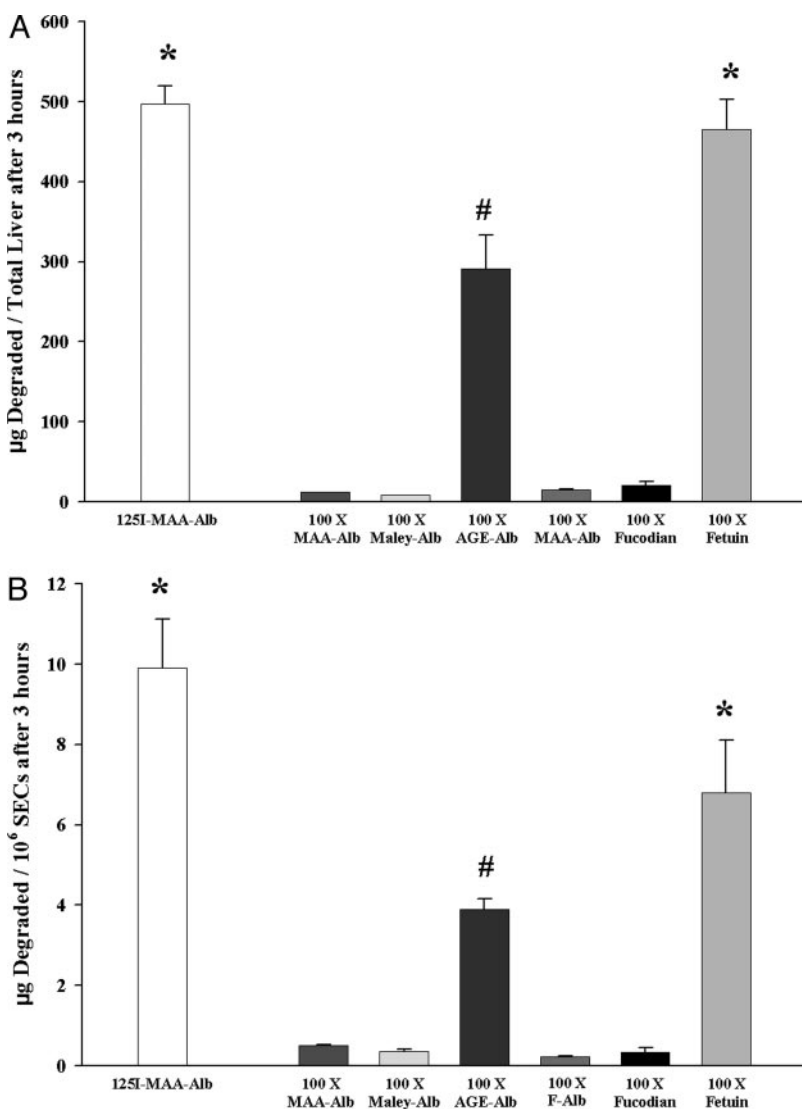


Fig. 2. Inhibition of ^{125}I -MAA-Alb degradation by in situ perfused livers (A) and isolated SECs (B) of chow-fed rats. Livers or isolated SECs were incubated for 3 h with 10 $\mu\text{g}/\text{ml}$ of ^{125}I -MAA-Alb and 100 fold concentrations of either unlabeled MAA-Alb, Maley-Alb, AGE-Alb, f-Alb, fucoidan, or fetuin. Intact protein was precipitated and acid soluble radioactivity (protein degradation) in the supernatant was determined. Results are expressed as means \pm S.D. ($n = 6$ in each group). *, $P < 0.001$ indicates significant differences from the ^{125}I -MAA-Alb alone. #, $P < 0.01$ indicates a significant difference from ^{125}I -MAA-Alb.

AGE-Alb, f-Alb, fucoidan, and the nonspecific polyanionic compound fetuin. As shown in Fig. 2A, 500 $\mu\text{g}/\text{ml}$ of ^{125}I -MAA-Alb is normally degraded over a 3-h period. However, when 100-fold unlabeled ligands were added, complete inhibition of degradation was observed for ^{125}I -MAA-Alb using MAA-Alb, Maley-Alb, f-Alb, and fucoidan. The degradation of ^{125}I -MAA-Alb was inhibited by only 40% when the AGE-Alb ligand was used, and the negative control fetuin had no inhibitory effects on the degradation of ^{125}I -MAA-Alb.

In previous studies, it was determined that MAA-Alb is degraded by isolated SECs of the liver (Duryee et al., 2003). Studies were performed to determine what scavenger receptor ligand would inhibit the degradation of ^{125}I -MAA-Alb using isolated SECs. ^{125}I -MAA-Alb was normally degraded at 10 $\mu\text{g}/\text{ml}$ after a 3-h period (Fig. 2B). When competing ligands were added, degradation of ^{125}I -MAA-Alb was completely inhibited with MAA-Alb, Maley-Alb, f-Alb, and fucoidan. Again, AGE-Alb would inhibit degradation by only 40%, and the fetuin control had no inhibitory effects.

Alcohol Effects on in Situ Degradation of ^{125}I -MAA-Alb. As previously reported by members of our laboratory (Thiele et al., 1999; Duryee et al., 2003), degradation of MAA-Alb and f-Alb is affected when rats are fed an alcohol diet. To begin looking at the effects of alcohol on the inhibition of these responses, rats were pair-fed the Lieber-DeCarli diet for 6 weeks, and inhibition of ^{125}I -MAA-Alb was assessed using 100-fold MAA-Alb, f-Alb, Maley-Alb, AGE-Alb, and fucoidan. Figure 3 shows that alcohol affects the amount of ^{125}I -MAA-Alb degraded over a 3-h period of in situ perfusion with the ligand (Duryee et al., 2003). When 100-fold of known scavenger receptor ligands were added to the perfusate, MAA-Alb, Maley-Alb, f-Alb, and fucoidan inhibited the response down by 100%. AGE-Alb, on the other hand, had only a 40% reduction in degradation. Fetuin, the negative control to fucoidan, had no inhibitory effects on the degradation of ^{125}I -MAA-Alb. The AGE-Alb inhibited MAA-Alb degradation in pair and ethanol-fed rats similar to the decreased effect seen by the alcohol.

RT-PCR Assessment of Scavenger Receptors. To begin examining more closely which scavenger receptors MAA-Alb might be using, chow-fed rats were injected with phosphate buffer, Alb, or MAA-Alb via the mesenteric vein, as described under *Materials and Methods*. After a 16-h incubation period, RNA from whole liver and SECs was isolated and subjected to RT-PCR using the primers listed in Table 1. As shown in Fig. 4, whole livers from animals injected with phosphate and Alb showed the expression of SRAI, SRBI, CD36, CD14, and glyceraldehyde-3-phosphate dehydrogenase mRNA. However, whole livers from animals injected with MAA-Alb decreased their expression of SRBI but increased the expression of LOX-1 mRNA.

When isolated SECs were examined for reactivity to scavenger receptors after MAA-Alb injection, LOX-1 and CD36 were increased (Fig. 4). In addition, the increased expression of TNF-alpha mRNA in SECs of MAA-Alb injected animals is consistent with findings from previous work performed in our laboratory (Duryee et al., 2004). Although these data are preliminary, they begin to provide some insight into what scavenger receptors may be involved in the binding and degradation of MAA adducted proteins.

SRA Knockout Mice. To further demonstrate that scavenger receptors are important for the uptake of MAA-Alb and the initiation of biological responses, SRA-KO mice were obtained. SRA-WT and SRA-KO mice were injected with 25 μg of Alb or MAA-Alb and a phosphate control as described under *Materials and Methods*. At the end of 6 weeks, SRA-WT mice exhibited little or no antibody response to the Alb or MAA-Alb when injected with phosphate or Alb (Fig. 5A). However, animals injected with MAA-Alb had antibody concentrations of 4000 $\mu\text{g}/\text{ml}$ in response to the Alb and 13,000 $\mu\text{g}/\text{ml}$ to the MAA-Alb antigens. When these same antigens were injected into SRA-KO mice (Fig. 5B), the concentration of antibody to the MAA adduct decreased to 7 $\mu\text{g}/\text{ml}$. Antibody to the Alb only, was similar in animals injected with all three antigens. There was an increase in

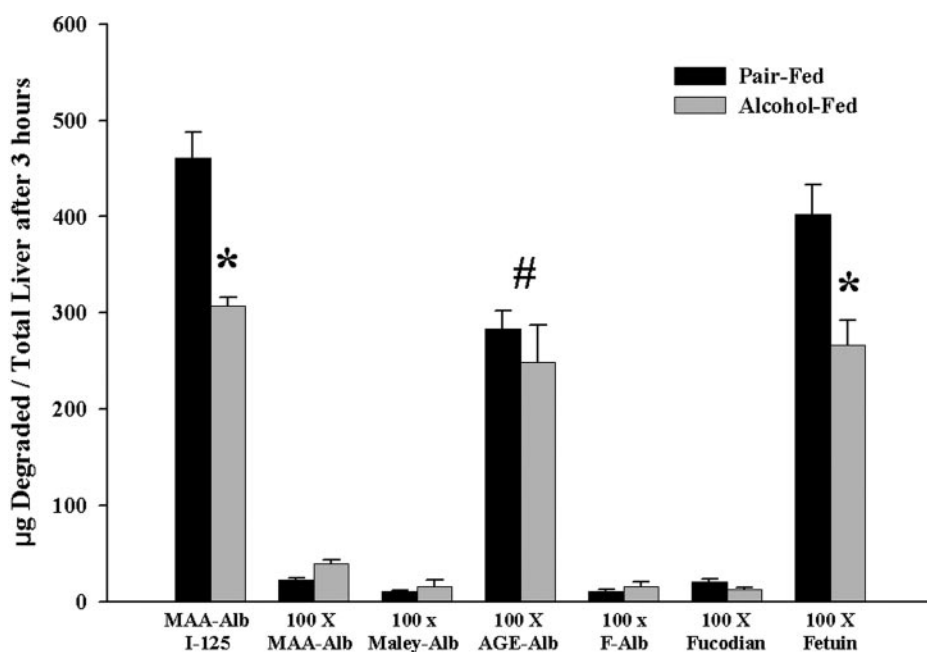


Fig. 3. Inhibition of ^{125}I -MAA-Alb degradation by in situ perfused livers from pair and alcohol-fed rats perfused with 10 $\mu\text{g}/\text{ml}$ MAA-Alb and inhibited with 100-fold concentrations of the following scavenger receptor antigens: MAA-Alb, Maley-Alb, AGE-Alb, f-Alb, fucoidan, and fetuin. Antigens were perfused for 3 h, intact protein was precipitated, and acid-soluble radioactivity (protein degradation) in the supernatant was determined. Results are expressed as means \pm S.D. ($n = 6$ in each group). *, $P < 0.01$ indicates significant differences from the pair-fed control animals. #, $P < 0.01$ indicates a significant difference from the pair-fed or chow-fed animals in MAA-Alb degradation.

MAA-Alb antibody over the phosphate control for animals injected with both Alb and MAA-Alb.

Discussion

Scavenger receptors have been shown to bind aldehyde-modified proteins (Brown et al., 1980; Horiuchi et al., 1986; Takata et al., 1988, 1989; Steinbrecher et al., 1989). These receptors are thought to provide a mechanism for the clearance of modified proteins from the circulation through a number of cell types, including SECs. Most recently, the aldehyde-modified protein MAA-Alb has been shown to bind and to be degraded by SECs (Duryee et al., 2003). To determine whether MAA-Alb enters these cells through scavenger receptors, inhibition studies were performed. These studies used known scavenger receptor ligands to inhibit the degradation of f-Alb and MAA-Alb in an in situ liver perfusion model. Data acquired from these studies showed that f-Alb degradation was completely inhibited using unlabeled f-Alb, Maley-Alb, AGE-Alb, and fucoidan. MAA-Alb inhibited the response by only 50%, indicating incomplete binding to the same receptor as f-Alb.

When isolated SECs were used in inhibition studies in vitro, similar results were observed. Studies were next designed to test MAA-Alb as the ligand and f-Alb, Maley-Alb, AGE-Alb, and fucoidan as the competing ligands. Data from these experiments demonstrated that MAA-Alb degradation was inhibited by MAA-Alb, Maley-Alb, f-Alb, and fucoidan. However, AGE-Alb would inhibit MAA-Alb degradation by

only 40%. These results were similar when isolated SECs were used in cell-specific assays. Recent work by Hansen et al. (2002b) showed that endocytosis of the AGE product may lead to the depletion of the scavenger receptor and result in the inability to clear these molecules from the circulation. In another study, Smedsrod (2004) speculated that oxidized low-density lipoprotein may be taken up by scavenger receptors, implicating the buildup of this product in atherosclerosis. In both situations, the result is the presence of potentially harmful products that could cause tissue damage. Recent studies have shown MAA-adducted proteins are associated with atherosclerosis-induced vascular inflammatory injury and could provide a mechanism by which MAA-adducts build up and/or down-regulate the receptor (Hill et al., 1998).

In experiments done previously by our laboratory (Thiele et al., 1996, 1999; Duryee et al., 2003), when ethanol was fed to rats, it decreased the degradation of acetaldehyde-Alb, f-Alb, and MAA-Alb by both in situ perfusion and isolated SECs. To determine whether inhibition of MAA-Alb would be affected by ethanol, rats were given alcohol as stated under *Materials and Methods* and subjected to in situ perfusion with ^{125}I -MAA-Alb and 100-fold inhibitors: MAA-Alb, Maley-Alb, AGE-Alb, f-Alb, and fucoidan. Data from these studies were similar to chow-fed rat data, in that MAA-Alb could be inhibited in both pair-fed and ethanol-fed rats with MAA-Alb, Maley-Alb, f-Alb, and fucoidan. Consistent with the chow-fed data, AGE-Alb inhibited the degradation of MAA-Alb by only 40%. It is interesting that alcohol reduced MAA-

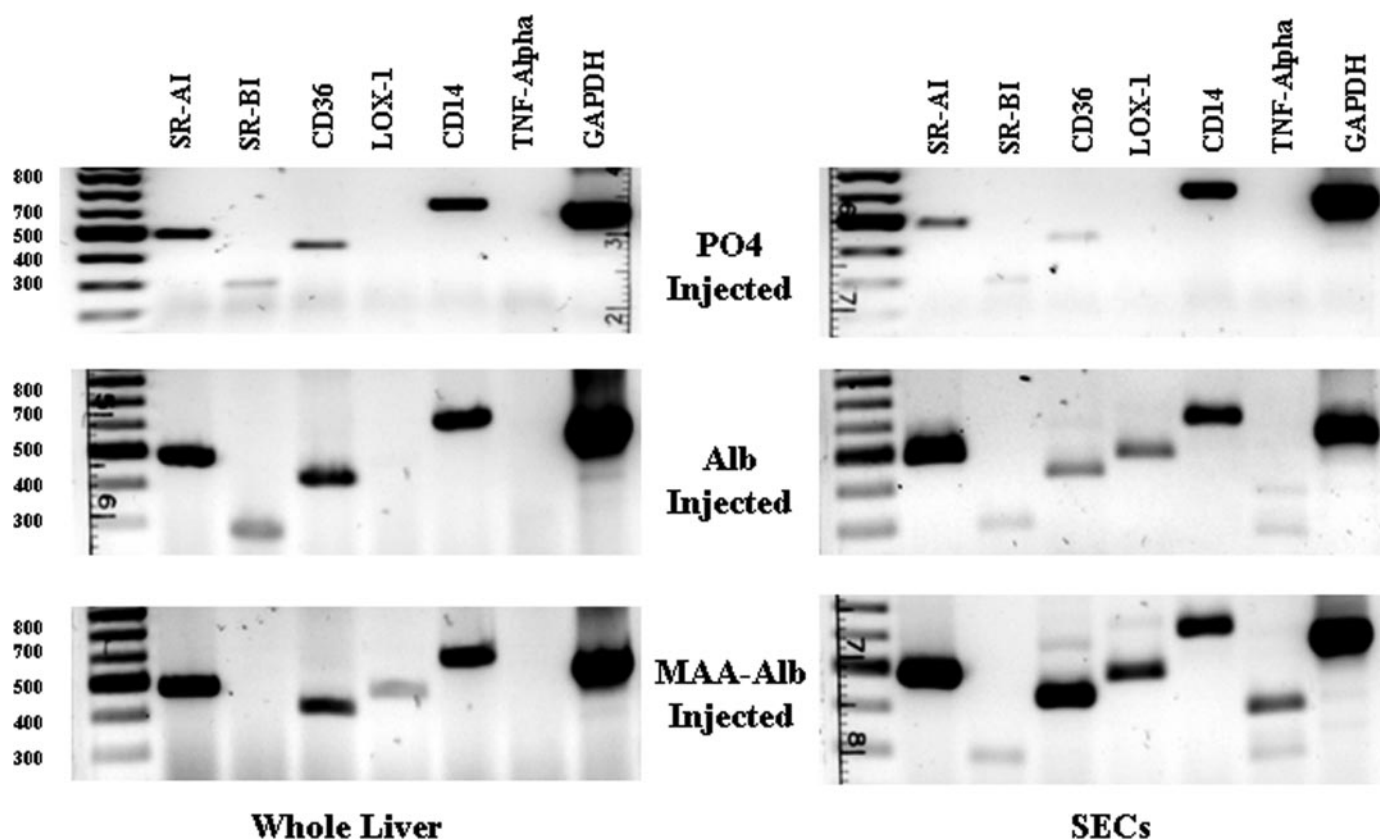


Fig. 4. Expression of scavenger receptors on SECs after mesenteric vein injection of MAA-Alb. Chow-fed rats were injected via the mesenteric vein with PBS, Alb, and MAA-Alb and incubated for 16 h, whole liver and SECs were isolated, RNA was purified, and RT-PCR was performed for SR-AI (495 bp), SR-BI (290 bp), CD36 (437 bp), LOX-1 (491 bp), CD14 (623 bp), TNF- α (351 bp), and glyceraldehyde-3-phosphate dehydrogenase (590 bp). Gels are representative of five animals injected with the above ligands.

Alb degradation by 200 μg /total liver, whereas AGE-Alb inhibition reduced MAA-Alb degradation to approximately the same level. It was found that a defect in the internalization step was the reason for less degradation of MAA-Alb after alcohol consumption (Duryee et al., 2003). It is possible that the combination of MAA-Alb and AGE-Alb work in a similar fashion and compete for a similar receptor. This may down regulate the receptor and result in incomplete degradation. Future studies will need to examine exactly how MAA-Alb and AGE-Alb are working to decrease this response.

The next step in these experiments was to determine whether these responses could be produced in an in vivo model by injecting the mesenteric vein with phosphate buffer, Alb, and MAA-Alb. Injection of the mesenteric vein was chosen to show these responses would occur in an in vivo model system. To assess these responses, RNA was subjected to RT-PCR using primers specific for the scavenger receptors: SR-AI, SR-BI, CD36, LOX-1, and CD14. TNF- α was used as a control, in accordance with the fact that MAA-Alb stimulation has been shown to increase levels of this cytokine (Duryee et al., 2004). Data from these studies show the presence of SR-AI, CD36, and CD14 consistently for both

whole liver and SECs. However, in the whole-liver samples from MAA-Alb-injected animals, SR-BI is decreased and LOX-1 is increased. When SECs were examined for their response, SR-BI, CD36, LOX-1, and TNF- α were increased in response to MAA-Alb injection. These data do not prove the specific receptor with which MAA-Alb interacts. However, the present data suggest that MAA-Alb is entering the cells through a number of different receptors depending on concentration, presence of other ligands, and receptor function. These data show the presence of scavenger receptor type A in whole liver and isolated SECs consistently. The fact there is no change in this receptor makes it one possible candidate for the binding and degradation of MAA modified proteins.

To begin looking at the SRA more specifically, SRA-KO mice were obtained. These mice were injected with phosphate, Alb, and MAA-Alb and then assayed for antibody to Alb or MAA-Alb. This MAA-Alb ligand has been shown to produce antibody to this adduct without the use of adjuvant (Thiele et al., 1998). If scavenger receptor A is involved in this process, then antibody to MAA-Alb should be decreased in the knockout animals. Data from these studies proved that MAA-Alb injection in the wild-type mice produced 13,000

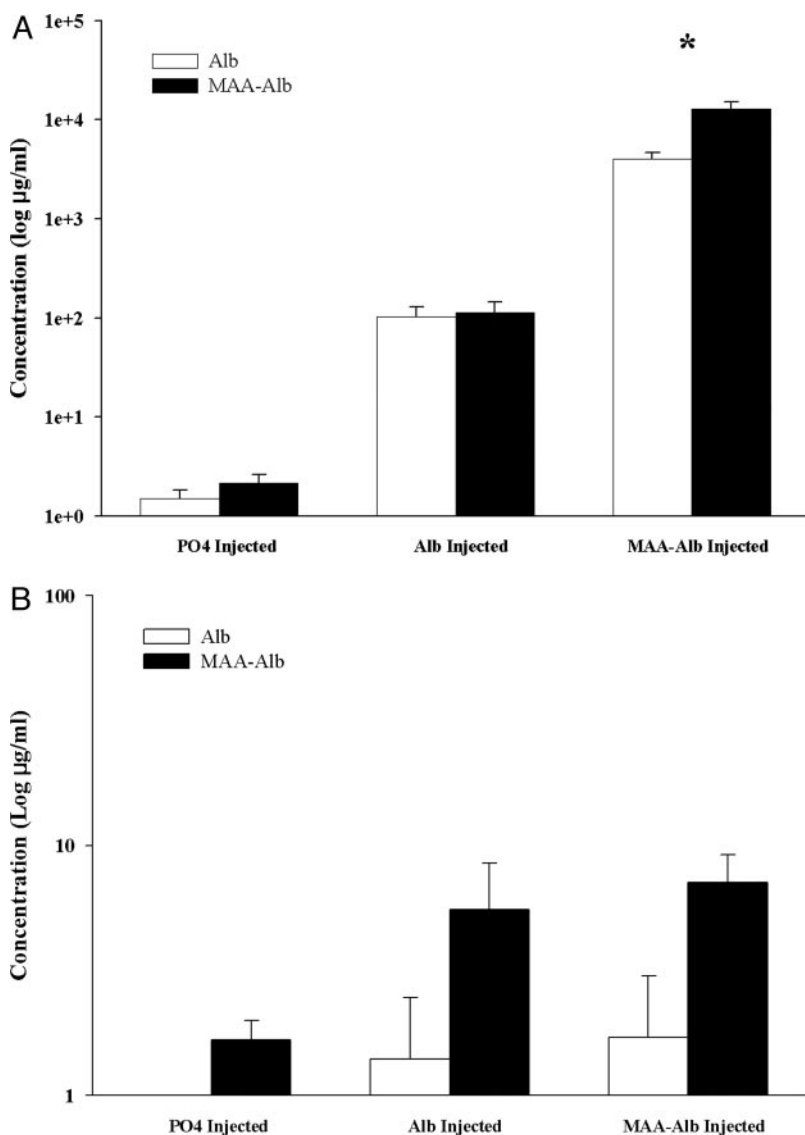


Fig. 5. Antibody response to MAA-Alb in scavenger receptor type a knockout mice. Wild-type (A) and knockout (B) mice were injected with 25 $\mu\text{g}/\text{ml}$ of Alb or MAA-Alb and a PBS control for 6 weeks and screened against Alb or MAA-Alb using a direct enzyme-linked immunosorbent assay. Results are reported as a decrease in the antibody response to MAA-Alb. Results are expressed as means in log format \pm S.D. ($n = 5$ in each group). *, $P < 0.01$

$\mu\text{g/ml}$ antibody, whereas injection in the knock mice reduced this number to $7 \mu\text{g/ml}$. This dramatic decrease in antibody to the MAA-Alb suggests that this ligand does enter cells via scavenger receptor type A. Because this antibody response could not be completely wiped out, the possibility still exists for other receptors to be involved in the processing of MAA-Alb. Data from the mesenteric vein injection of MAA-Alb suggests that LOX-1 or CD36 may be involved to a certain degree in these processes. Experiments involving Chinese hamster ovarian cells expressing the different scavenger receptors are in process within our laboratory to determine more specifically which receptors are involved in the processing of MAA-Alb.

In summary, degradation of f-Alb is inhibited with f-Alb, Maley-Alb, AGE-Alb, and fucoidan. MAA-Alb was the only ligand to not completely inhibit f-Alb degradation in both in situ perfusion and isolated SECs. When MAA-Alb was used as the ligand, its degradation was inhibited with MAA-Alb, f-Alb, Maley-Alb, and fucoidan. AGE-Alb was the only modified protein to not completely inhibit MAA-Alb degradation in both in situ perfusion and isolated SECs. When alcohol was fed to rats and in situ liver perfusion was performed, results similar to those in the chow-fed data were observed. The incomplete inhibition with AGE-Alb was similar to the numbers obtained with alcohol use on its own. RT-PCR analysis for scavenger receptors demonstrated that MAA-Alb would decrease SR-BI and increase LOX-1 in whole liver after MAA-Alb injection and increase LOX-1 and CD36 in isolated SECs. The fact that scavenger receptor A knockout mice experiments showed a decreased MAA-Alb antibody production suggests that SR-AI may be a significant player in the binding and degradation of MAA-Alb.

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References

Abraham R, Choudhury A, Basu SK, Bal V, and Rath S (1997) Disruption of T cell tolerance by directing a self antigen to macrophage-specific scavenger receptors. *J Immunol* **158**:4029–4035.

Abraham R, Singh N, Mukhopadhyay A, Basu SK, Bal V, and Rath S (1995) Modulation of immunogenicity and antigenicity of proteins by maleylation to target scavenger receptors on macrophages. *J Immunol* **154**:1–8.

Alaiz M, Beppu M, Ohishi K, and Kikugawa K (1994) Modification of delipidated apoprotein B of low density lipoprotein by lipid oxidation products in relation to macrophage scavenger receptor binding. *Biol Pharm Bull* **17**:51–57.

Araki N, Higashi T, Mori T, Shibayama R, Kawabe Y, Kodama T, Takahashi K, Shichiri M, and Horiuchi S (1995) Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. *Eur J Biochem* **230**:408–415.

Barnes JL, Reznicek MJ, Radnik RA, and Venkatachalam MA (1988) Anionization of an antigen promotes glomerular binding and immune complex formation. *Kidney Int* **34**:156–163.

Braet F, De Zanger R, Sasaoki T, Baekelandt M, Janssens P, Smedsrod B, and Wisse E (1994) Assessment of a method of isolation, purification and cultivation of rat liver sinusoidal endothelial cells. *Lab Invest* **70**:944–952.

Brown MS, Basu SK, Falck JR, Ho YK, and Goldstein JL (1980) The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. *J Supramol Struct* **13**:67–81.

Duryee MJ, Klassen LW, Freeman TL, Willis MS, Tuma DJ, and Thiele GM (2003) Chronic ethanol consumption impairs receptor-mediated endocytosis of MAA-modified albumin by liver endothelial cells. *Biochem Pharmacol* **66**:1045–1054.

Duryee MJ, Klassen LW, Freeman TL, Willis MS, Tuma DJ, and Thiele GM (2004) Lipopolysaccharide is a cofactor for malondialdehyde-acetaldehyde adduct-

mediated cytokine/chemokine release by rat sinusoidal liver endothelial and Kupffer cells. *Alcohol Clin Exp Res* **28**:1931–1938.

Hansen B, Melkko J, and Smedsrod B (2002a) Serum is a rich source of ligands for the scavenger receptor of hepatic sinusoidal endothelial cells. *Mol Cell Biochem* **229**:63–72.

Hansen B, Svistunov D, Olsen R, Nagai R, Horiuchi S, and Smedsrod B (2002b) Advanced glycation end products impair the scavenger function of rat hepatic sinusoidal endothelial cells. *Diabetologia* **45**:1379–1388.

Hill GE, Miller JA, Baxter BT, Klassen LW, Duryee MJ, Tuma DJ, and Thiele GM (1998) Association of malondialdehyde-acetaldehyde (MAA) adducted proteins with atherosclerotic-induced vascular inflammatory injury. *Atherosclerosis* **141**:107–116.

Hoff HF and O'Neil J (1993) Structural and functional changes in LDL after modification with both 4-hydroxynonenal and malondialdehyde. *J Lipid Res* **34**:1209–1217.

Horiuchi S, Murakami M, Takata K, and Morino Y (1986) Scavenger receptor for aldehyde-modified proteins. *J Biol Chem* **261**:4962–4966.

Kikugawa K and Ido Y (1984) Studies on peroxidized lipids. V. Formation and characterization of 1,4-dihydropyridine-3,5-dicarbaldehydes as model of fluorescent components in lipofusion. *Lipids* **19**:464–475.

Lieber CS and DeCarli LM (1982) The feeding of alcohol in liquid diets: two decades of applications and 1982 update. *Alcohol Clin Exp Res* **6**:523–531.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275.

McConahey PJ and Dixon FJ (1980) Radioiodination of proteins by the use of the chloramine-T method. *Methods Enzymol* **70**:210–213.

Mego JL, Bertini F, and McQueen JD (1967) The use of formaldehyde-treated 131-I-albumin in the study of digestive vacuoles and some properties of these particles from mouse liver. *J Cell Biol* **32**:699–707.

Nicoletti A, Caligiuri G, Tornberg I, Kodama T, Stemme S, and Hansson GK (1999) The macrophage scavenger receptor type A directs modified proteins to antigen presentation. *Eur J Immunol* **29**:512–521.

Ogami N, Nagai R, Ikemoto M, Arai H, Kuniyasu A, Horiuchi S, and Nakayama H (2001a) CD36, a member of class B scavenger receptor family, is a receptor for advanced glycation end products. *Ann NY Acad Sci* **947**:350–355.

Ogami N, Nagai R, Miyazaki A, Ikemoto M, Arai H, Horiuchi S, and Nakayama H (2001b) Scavenger receptor class B type I-mediated reverse cholesterol transport is inhibited by advanced glycation end products. *J Biol Chem* **276**:13348–13355.

Palinski W, Yla-Herttuala S, Rosenfeld ME, Butler SW, Socher SA, Parthasarathy S, Curtiss LK, and Witztum JL (1990) Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis* **10**:325–335.

Smedsrod B (2004) Clearance function of scavenger endothelial cells. *Comp Hepatol* **3** (Suppl 1):S22.

Steinbrecher UP, Loughheed M, Kwan WC, and Dirks M (1989) Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *J Biol Chem* **264**:15216–15223.

Suzuki H, Kurihara Y, Takeya M, Kamada N, Kataoka M, Jishage K, Ueda O, Sakaguchi H, Higashi T, Suzuki T, et al. (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature (Lond)* **386**:292–296.

Takata K, Horiuchi S, Araki N, Shiga M, Saitoh M, and Morino Y (1988) Endocytic uptake of nonenzymatically glycosylated proteins is mediated by a scavenger receptor for aldehyde-modified proteins. *J Biol Chem* **263**:14819–14825.

Takata K, Horiuchi S, Araki N, Shiga M, Saitoh M, and Morino Y (1989) Scavenger receptor of human monocytic leukemia cell line (THP-1) and murine macrophages for nonenzymatically glycosylated proteins. *Biochim Biophys Acta* **986**:18–26.

Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, and van Berkel TJ (2000) Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler Thromb Vasc Biol* **20**:1860–1872.

Thiele GM, Miller JA, Klassen LW, and Tuma DJ (1996) Long-term ethanol administration alters the degradation of acetaldehyde adducts by liver endothelial cells. *Hepatology* **24**:643–648.

Thiele GM, Miller JA, Klassen LW, and Tuma DJ (1999) Chronic ethanol consumption impairs receptor-mediated endocytosis of formaldehyde-treated albumin by isolated rat liver endothelial cells. *Hepatology* **29**:1511–1517.

Thiele GM, Tuma DJ, Willis MS, Miller JA, McDonald TL, Sorrell MF, and Klassen LW (1998) Soluble proteins modified with acetaldehyde and malondialdehyde are immunogenic in the absence of adjuvant. *Alcohol Clin Exp Res* **22**:1731–1739.

Tuma DJ, Thiele GM, Xu D, Klassen LW, and Sorrell MF (1996) Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration. *Hepatology* **23**:872–880.

Uchida K (2000) Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med* **28**:1685–1696.

Vlassara H, Brownlee M, and Cerami A (1985) High-affinity-receptor-mediated uptake and degradation of glucose-modified proteins: a potential mechanism for the removal of senescent macromolecules. *Proc Natl Acad Sci USA* **82**:5588–5592.

Yang Z, Makita Z, Horii Y, Brunelle S, Cerami A, Sehajpal P, Suthanthiran M, and Vlassara H (1991) Two novel rat liver membrane proteins that bind advanced glycosylation endproducts: relationship to macrophage receptor for glucose-modified proteins. *J Exp Med* **174**:515–524.

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