Scavenger receptors on liver Kupffer cells mediate the in vivo uptake of oxidatively damaged red blood cells in mice

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In vitro studies have shown that damaged red cells and apoptotic cells are efficiently phagocytosed by scavenger receptors from macrophages, even under non-opsonizing conditions. Damaged red blood cells are in vivo effectively removed from the blood circulation, but the responsible receptor systems are largely unknown. We used a murine model in which 51Cr-labeled oxidized red blood cells were injected intravenously, and the cellular uptake sites and the potential involvement of scavenger receptors were analyzed. The decay of damaged red cells was rapid (more than 50% removed within 10 minutes after injection), whereas native red cells were not cleared. The main site of uptake of damaged red cells was the liver Kupffer cells, which contained 24% of the injected dose at 10 minutes after injection. The blood decay and liver uptake were inhibited by typical ligands for scavenger receptors, such as polyinosinic acid, liposomes containing phosphatidylserine, oxidized low-density lipoprotein, and fucoidan, but not by polyadenosinic acid or liposomes without phosphatidylserine. Mice lacking scavenger receptors class A type I and II showed no significant decrease in the ability to take up damaged red cells from the circulation. We conclude that Kupffer cells are mainly responsible for the removal of damaged red cells from the blood circulation, a process mediated by polyinosinic acid- and phosphatidylserine-sensitive scavenger receptors, different from scavenger receptor class A type I and II. Our data indicate that scavenger receptors, as pattern-recognition receptors, play an important role in vivo in the removal of apoptotic, damaged, or other unwanted cells from the blood circulation.

Introduction

It is generally accepted that recognition of aged, infected, or damaged red blood cells (RBCs) is mediated by pathways that include altered carbohydrate moieties of membrane proteins, adherence of antibody 3 autoantibodies, or the loss of membrane phospholipid asymmetry. To what extent these changes are involved in providing a signal for erythrophagocytosis in vivo is not clear. During normal aging of RBCs, auto-oxidative damage occurs to lipid and protein components in the membrane. Previous in vitro studies showed that the binding and phagocytosis of RBCs, which were oxidatively damaged (OxRBC), can be inhibited by ligands for macrophage scavenger receptors. Oxidized low-density lipoprotein (OxLDL), but not native or acetylated low-density lipoprotein (LDL), inhibited the binding of OxRBC to isolated murine peritoneal macrophages by approximately 80%. Other scavenger receptor ligands, such as fucoidan, polyinosinic acid (poly I), and liposomes containing phosphatidylserine (PS), exhibited similar inhibitory properties, and it was thus suggested that OxRBC are a possible ligand for scavenger receptors. This suggestion is consistent with the hypothesis that scavenger receptors, as pattern-recognition receptors, are involved in the innate immune system in which macrophages play a role in the discrimination between “self” and “non-self.” The removal of apoptotic, damaged, and other unwanted cells from the blood circulation or tissues is important for homeostasis, and any impairment of this process could potentially result in chronic inflammation.

Apart from scavenger receptors, numerous other receptors have been described that may participate in recognition and phagocytosis by macrophages. The macrophage mannose receptor recognizes exposed mannose residues of plasma membrane proteins, and F and complement receptors are responsible for the uptake of opsonized particles. However, many studies have shown that even under non-opsonizing conditions, damaged cells, such as apoptotic cells or senescent erythrocytes, are efficiently taken up by macrophages. This recognition can be mediated through progressive exposure of PS on the outer leaflet of the plasma membrane during aging, damage, or apoptosis.

The potential function and quantitative role of scavenger receptors for the removal of modified cells in vivo has not been studied, and our data are aimed to show the potential relevance of the earlier in vitro data for the in vivo situation. In vivo, many different receptors or different cell types in various organs may operate simultaneously, and their activity will depend on receptor expression levels, on cellular localization, as well as on the presence of serum components or extracellular matrix. The present studies were undertaken to analyze the cell type(s) responsible for the in vivo uptake of OxRBC and the potential involvement in vivo of scavenger receptors. In addition to normal mice, scavenger receptor class A (SRA) knock out mice were utilized to verify to what extent this well-characterized receptor system might be responsible for OxRBC removal.
Materials and methods

Materials

51Cr-sodium chromate was from Amersham (Buckinghamshire, UK), CuSO4, L-ascorbic acid, poly I, polyadenosinic acid (poly A), fucoidan, collagenase (type IV), bovine serum albumin (BSA, fraction V), 3,3'-diaminobenzidine, Mayer’s haematoxylin solution, and cholesterol were from Sigma (St. Louis, MO). Bovine brain PS and egg yolk phosphatidylcholine (PC) were purchased from Fluka (Buchs, Switzerland); Hepes was from Biosolve (Valkenswaard, The Netherlands), and EDTA was from Merck (Darmstadt, Germany).

Mice

Six- to 10-week-old male ICR mice with a weight between 26 and 32 grams were used. In some experiments, these mice were compared with male SV129 × ICR mice of the approximate age and weight. The latter lack expression of SRA I and II by disruption of exon 4 of the gene and were a kind gift from Dr Suzuki et al. Mice were bred in our local facility and kept under low pathogen conditions with free access to food and drink.

RBC preparation

Venous blood was drawn from a male, wild-type ICR mouse with 10 U/mL heparin. The RBC pellet was washed 4 times with sterile phosphate-buffered saline (PBS) and kept as a 20% hematocrit suspension. RBCs were used within 3 days of storage. Labeling was carried out with 50 µCi 51Cr-sodium chromate per mL of a 10% hematocrit RBC suspension in sterile PBS for 30 minutes at 37°C while gently shaking. RBCs were washed at least 4 times to remove free label and immediately incubated with 200 µmol/L CuSO4 in PBS containing 5 mmol/L ascorbic acid for 90 minutes at 37°C. Oxidation by Cu²⁺/ascorbic acid leads to extensive membrane crosslinking, as shown by SDS/PAGE, which is comparable to treatment of RBCs with 1 mmol/L glutaraldehyde or 200 µmol/L CuSO4 plus 10 mmol/L H2O2. The RBCs showed a 4-fold increase in methemoglobin, as compared with untreated cells. Peroxide treatment or aldehyde treatment was shown earlier to enhance phagocytosis of RBCs. After oxidation, RBCs were washed once with PBS/5 mmol/L EDTA and once with PBS alone. A volume of 300 µL of a 10% hematocrit suspension was counted on a gamma counter (5550 Minaxi gamma, Packard, Downers Grove, IL) with a window set for 51Cr (200-400 KeV) to calculate the total injected dose afterward.

Liver uptake, blood decay, and tissue distribution of RBCs in mice

Mice were anesthetized with a subcutaneous injection of a mixture of Nembutal (112.5 mg ketamine/kg body weight), Hypnorm (1.125 mg fluanisone/kg and 0.035 mg fentanyl citrate/kg), and Thalamonal (1.6 mg droperidol/kg and 0.032 mg fentanyl/kg). The abdomens were opened, and 200 µL of a 10% hematocrit suspension of 51Cr-labeled RBCs (± 3.5 × 10⁸ RBCs) were injected via the cava vein or the tail vein with or without preinjection of competitors. At the indicated times, liver lobules were tied off, excised, and weighed. The amount of liver tissue tied off during the experiment did not exceed 10% of the total liver weight. Blood samples (<50 µL) were taken, and radioactivity was measured. At 30 minutes, the mice were killed and tissues were excised and weighed to determine the tissue distribution. The radioactivity in liver and other tissue samples was corrected for blood present at the time of sampling, according to earlier determinations with 51Cr-labeled RBCs in these mice. The total blood volume was subsequently with 100 mL of collagenase buffer (67 mmol/L NaCl/6.7 mmol/L KCl/6.7 mmol/L Hepes/2% BSA) containing 20 mg collagenase type VI, pH 7.6. Parenchymal, endothelial, and Kupffer cells were isolated by differential centrifugation and counterflow elutriation, as described earlier for rats. The different cell fractions were stained with 3,3'-diaminobenzidine for endogenous peroxidase activity and analyzed by light microscopy. The percentage of cells containing RBCs was determined by counting. To characterize the nonparenchymal cells, cyto- spins of each cell fraction were prepared. Cytospins were fixed with cold acetone and stained with haematoxylin solution.

Macrophage depletion

Liposomes containing dichloromethylene diphosphonate (DMDP) were a kind gift from Dr N. van Rooijen (Vrije Universiteit, Amsterdam). A volume of 200 µL liposome suspension (± 5 mg DMDP/mL) was injected into the tail vein 48 hours prior to the injection of RBCs. This treatment has been shown to deplete the liver and the spleen of most of the present macrophages. Control mice had the same volume of sterile PBS injected.

Liposomes

Unilamellar liposomes were prepared with egg yolk PC, bovine brain PS, and cholesterol in a phospholipid-to-cholesterol molar ratio of 2:1. Lipids in chloroform were mixed and evaporated under nitrogen. The lipid film was resuspended in sterile PBS at a concentration of 3 mmol/L total lipid and sonicated for 30 minutes with an MSE soniprep 150 (amplitude 16 µ) at 52°C under a constant stream of argon. The average particle size (47.0 nm for PC/cholesterol liposomes and 55 nm for PS/PC/cholesterol) and homogeneity were measured by photon correlation spectroscopy (System 4700 C, Malvern Instruments, Malvern, UK).

Lipoprotein preparation

LDL was isolated from serum by differential ultracentrifugation. Oxidation of LDL (0.1 mg/mL) was carried out with 10 µmol/L CuSO4 in PBS for 18 hours. The preparation was concentrated by speed vacuum rotation, and the protein concentration was measured by the method of Lowry.

Statistical analysis

Data are shown as mean (± SEM). Statistical significance was calculated with 1-way analysis of variance.

Results

RBC clearance and tissue distribution

RBCs were isolated, labeled with 51Cr-sodium chromate, and injected into recipient mice via the cava vein or the tail vein. Without oxidative treatment, 51Cr-labeled RBCs were not taken up by any tissue, and, even at 24 hours after injection, the injected dose could be completely recovered from the blood. However, introduction of RBCs treated with CuSO4 and ascorbic acid resulted in a rapid blood decay and uptake by various tissues (Figure 1 A-C), mainly by the liver and spleen. At 30 minutes after injection, more than 70% of the injected OxRBC were removed from the circulation, whereby 31% is taken up by the liver and 15% by the spleen. The relatively high association of OxRBC with the lungs at 30 minutes after injection appeared to be transient, since at 24 hours, a low activity was observed. Injection of OxRBC into the portal vein as route of entry resulted in an increase in liver uptake and a concomitant decrease in the lungs, which indicates that part of the uptake in the lungs is due to temporary trapping of the cells in the capillary bed (data not shown).
I-TC-AcLDL per mg of cell protein, whereas without DMDP-liposomes this value was 6.99 ± 1.63% of the injected dose per mg cell protein. With preadministration of DMDP-liposomes, liver uptake of OxRBC by the liver was inhibited by more than 80%, whereas PC liposomes had no significant effect on the blood removal. The uptake of 51Cr-OxRBC by the liver was inhibited by more than 80%, whereas poly I, fucoidan, and OxLDL, on the liver and spleen uptake (Figure 5). Of the ligands tested, poly I showed a highly significant inhibition, both on the clearance of OxRBC from the blood circulation (not shown) and on the uptake by the liver and spleen, whereas poly A was ineffective. OxLDL was also effective in inhibiting both liver and spleen uptake of OxRBC by about 50%, similar to fucoidan (Figure 5).

Tissue distribution of OxRBC in SRA knockout mice

From the competition experiments, we learned that typical scavenger receptor ligands could efficiently compete for the uptake of OxRBC in vivo. However, no definitive conclusions regarding the identity of the particular types of scavenger receptors involved could be drawn. There is considerable overlap in ligand binding properties among the various types of scavenger receptors. To determine whether the lack of SRA on Kupffer cells influenced the uptake of OxRBC, we compared the in vivo fate of OxRBC in SRA knockout and control animals. Furthermore, no difference in the rate of blood decay nor the liver uptake of OxRBC. We found that the rate of blood decay is decreased considerably by the presence of liposomes containing PS (Figure 4A). Liposomes without PS (PC liposomes) had no significant effect on the blood removal. The uptake of 51Cr-OxRBC by the liver was inhibited by more than 80%, whereas PC liposomes were much less effective (Figure 4B and C). We also examined the effect of other ligands for scavenger receptors such as poly I, fucoidan, and OxLDL, on the liver and spleen uptake (Figure 5). Of the ligands tested, poly I showed a highly significant inhibition, both on the clearance of OxRBC from the blood circulation (not shown) and on the uptake by the liver and spleen, whereas poly A was ineffective. OxLDL was also effective in inhibiting both liver and spleen uptake of OxRBC by about 50%, similar to fucoidan (Figure 5).

Discussion

The removal of damaged and dying cells from the blood circulation and tissues is important for the maintenance of cellular homeostasis.
Figure 2. Light microscopy of nonparenchymal liver cells for the presence of oxidatively damaged red blood cells at 30 minutes after injection. Cells were isolated as described in “Materials and methods.” Cytospins were fixed in acetone, stained with 3,3′-diaminobenzidine for endogenous peroxidase activity, and counterstained with hematoxylin. Red blood cells are dark brown. (A, B) Cells eluted at a flow rate of 26 mL/min (liver endothelial cells). (C, D) Cells eluted at a flow rate of 75 mL/min (Kupffer cells). Objective magnification ×40 (A, C); ×100 (B, D). The procedure was repeated 3 times and gave very similar results.
The recognition of damaged cells by different macrophage populations has mainly been studied by in vitro experiments, but information on their removal from the blood circulation and their tissue fate is lacking. To determine the relevance of the in vitro observation for the in vivo situation, we used mice in which 51Cr-labeled oxidized RBCs were injected intravenously, and the organ and cellular uptake sites were quantitatively analyzed (the recovery of the injected label was 86.4 ± 4.3%). In addition, the characteristics of the site(s) responsible for OxRBC removal were analyzed by in vivo competition studies with PS and PC liposomes, OxLDL, fucoidan, and polyanions. Furthermore, the potential involvement of the likely candidate receptor, SRA type I,II, was analyzed by using mice deficient for this particular receptor. On injection into mice, 51Cr-labeled murine OxRBC were rapidly removed from the blood circulation, whereas native RBCs, as anticipated, remained in the circulation. Within 10 minutes after injection, more than 50% of the injected OxRBC were removed from the blood circulation and 24% were found in the liver. At 30 minutes, 31% of the injected cells were present in the liver and 15% in the spleen. It thus appears that the liver is the major organ for OxRBC clearance, showing a 2-fold higher uptake than the spleen. However, it must be realized that, on a weight basis, the spleen uptake greatly exceeds the liver uptake per gram tissue, a finding reminiscent of the clearance of PS-bearing red cells. Light microscopic analysis of the isolated liver cells at 30 minutes after injection of OxRBC indicated that 65% of the isolated Kupffer cells had ingested 1 or more RBC, and there was no evidence for uptake of OxRBC by liver endothelial cells. When Kupffer cells were depleted from the mice by pretreatment with DMDP-liposomes, no uptake of OxRBC occurred by the liver and only very little by the spleen. These data indicate that Kupffer cells and spleen macrophages are largely responsible for the removal of OxRBC from the blood circulation.

In vitro data with peritoneal macrophages have indicated that the interaction of oxidatively damaged erythrocytes with macrophages is mediated by a macrophage receptor with specificity for OxLDL. To analyze whether specific receptors on Kupffer cells and spleen macrophages are involved in the removal of OxRBC in intact mice, we determined whether ligands for scavenger receptors interfere with the biological fate of the cells. It appears that the liver uptake of OxRBC is competed for by typical ligands for scavenger receptors, namely poly I, liposomes containing PS, fucoidan, and OxLDL. PS liposomes efficiently blocked the liver uptake of OxRBC compared with control liposomes. This blockage led to a significant inhibition of the removal of OxRBC from the blood circulation. Remarkably, the spleen uptake of the OxRBC was not influenced by the preinjection of PS liposomes. This finding indicates that different receptor system(s) are responsible for the removal of OxRBC by the liver and by the spleen. In contrast, preinjection of poly I, fucoidan, and OxLDL significantly inhibited uptake of OxRBC by both liver and spleen. Taken together, these results indicate that, in vivo, specific receptors with scavenger receptor-like properties are responsible for the removal of OxRBC from the blood circulation by liver Kupffer cells and spleen macrophages.

Among the various scavenger receptors, the SRA was the first to be fully characterized. The availability of SRA knockout mice made it possible to evaluate the importance of this receptor for the removal of OxRBC from the blood circulation. Apparently this receptor plays only a minor role, if any, in this process, since both the blood decay and the uptake by liver and other tissues of OxRBC in the control and knockout animals were largely similar. This finding is in agreement with in vitro data, showing that acetylated LDL, a typical ligand for SRA, does not compete for the binding of OxRBC by peritoneal macrophages.

We have concentrated on the possibility that scavenger receptors are involved in the process of damaged cell removal, since some scavenger receptors show binding of anionic phospholipids and the exposure of PS on the outer leaflet of the plasma membrane is thought to be an important recognition site for removal of apoptotic cells by macrophages. It seems unlikely that MARCO, a scavenger receptor that is structurally related to SRA, would be involved, since it is not expressed on Kupffer cells in a nonactivated state. Another receptor recently implicated in the recognition of apoptotic cells is CD14, the LPS-receptor. Previous studies found that the 61D3 antibody, which has since been shown to be directed against CD14, inhibited the binding of apoptotic cells and phospholipid symmetric RBCs to
macrophages. Because fucoidan and phospholipids are also competitors for binding of LPS to CD14, this receptor is, therefore, a candidate for the recognition of OxRBC. The general availability of the CD14 knockout mice will be needed to unequivocally prove or disprove this possibility. The class B scavenger receptors, SR-BI and CD36, could also be involved, since they both have affinity for anionic phospholipids and CD36 recognizes RBCs infected with Plasmodium falciparum and apoptotic cells. Recently a lectin-like receptor for OxLDL, LOX-1, was cloned on the liver and spleen uptake of oxidatively damaged red blood cells (OxRBC). At 2 minutes before the injection of OxRBC, the indicated inhibitors were injected at the following amounts: polyadenosinic acid and polyinosinic acid: 200 µg (n = 3 ± SEM); fucoidan: 15 mg (n = 2 ± SEM); OxLDL: 150 µg protein (n = 2 ± SEM). *P < .05; **P < .001.

Figure 5. The effect of polyanions, fucoidan, or oxidized low-density lipoprotein (OxLDL) on the liver and spleen uptake of oxidatively damaged red blood cells (OxRBC). Liver (black bars) and spleen (hatched bars) uptake after injection of OxRBC. *P < .11.

Figure 6. Tissue distribution of oxidatively damaged red blood cells (OxRBC). Tissue distribution of OxRBC at 30 minutes after injection in wild-type (hatched bars) and scavenger receptor class A (SRA) knockout (black bars) mice. Shown are the means (n = 6 for wild-type mice, n = 3 for SRA knockout mice) ± SEM. *P = .13; **P = .11.

macrophages can be completely blocked in vivo by substrates for scavenger receptors. This blockade indicates that the recognition system for OxRBC on Kupffer cells and spleen macrophages shows scavenger receptor-like properties, although the studies with SRA knockout mice indicate a mechanism that is not dependent on SRA. We are currently investigating whether the in vivo clearance of apoptotic lymphocytes follows the same patterns as described here for OxRBC. Because the clearance of effete cells is of great importance for tissue homeostasis, it is possible that more than one member of the scavenger receptor family is involved in this pathway. Further characterization of the relative importance of the individual members of this family for the removal of aged cells forms an interesting future challenge.

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References


16. Takizawa F, Tsuji S, Nagasawa S. Enhancement


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