Lactoferrin Binding to Heparan Sulfate Proteoglycans and the LDL Receptor-Related Protein

Further Evidence Supporting the Importance of Direct Binding of Remnant Lipoproteins to HSPG

Zhong-Sheng Ji, Robert W. Mahley

Abstract Bovine lactoferrin inhibits the clearance of remnant lipoproteins from the plasma and competes with the cell-surface binding of apolipoprotein (apo) E-enriched remnants. We established that lactoferrin inhibits remnant binding and uptake by interacting with both heparan sulfate proteoglycans (HSPG) and the low-density lipoprotein receptor-related protein (LRP). The binding of 125I-lactoferrin was inhibited 45% to 60% in HepG2 hepatocytes and wild-type Chinese hamster ovary (CHO) cells treated with heparinase to remove HSPG. In mutant CHO cells (pgsD-677) lacking HSPG, the level of 125I-lactoferrin binding was ~50% that seen with wild-type CHO cells; thus, about one half of lactoferrin binding appears to be mediated through cell-surface HSPG. A significant fraction of the residual binding of the lactoferrin appears to be mediated through the LRP. The 39-kd protein known to bind to the LRP and to block ligand interaction inhibited 125I-lactoferrin degradation in wild-type CHO cells by 60% to 65%. The addition of the 39-kd protein plus heparinase treatment reduced the binding by 85% to 90% (this combination blocks direct interaction with both the LRP and HSPG). However, it was also shown that the 39-kd protein bound to HSPG and the LRP. Heparinase treatment of wild-type CHO cells decreased the binding of the 125I-39-kd protein by ~40%, and the mutant CHO cells lacking HSPG bound half as much 125I-39-kd protein as wild-type CHO cells. These studies also helped to establish that most of the enhanced binding of apoE-enriched β-very-low-density lipoproteins (β-VLDL) was via HSPG and not as a direct interaction with the LRP in the absence of HSPG. Whereas apoE-enriched β-VLDL at a high concentration inhibited ~45% of 125I-lactoferrin binding to wild-type CHO cells, 125I-lactoferrin binding to mutant CHO cells lacking HSPG (apparently binding to the LRP) was not inhibited by apoE-enriched β-VLDL, thus further suggesting that apoE-enriched β-VLDL does not interact to a major extent directly with the LRP in the absence of HSPG. (Arterioscler Thromb. 1994;14:2025-2032.)

Key Words • apolipoprotein E • chylomicron metabolism • β-VLDL

The intravenous injection of bovine lactoferrin in rats partially inhibits the clearance of chylomicron remnants by 50% to 75%. Furthermore, the decreased clearance caused by lactoferrin appears to be secondary to the inhibition of uptake specifically by hepatocytes in the rat liver. On ligand blots, lactoferrin binds to the low-density lipoprotein (LDL) receptor-related protein (LRP), a receptor that appears to participate in remnant lipoprotein uptake. Furthermore, lactoferrin blocks the uptake of apolipoprotein (apo) E-enriched β-very-low-density lipoproteins (β-VLDL) by human fibroblasts lacking LDL receptors. In addition, lactoferrin partially inhibits the uptake of β-VLDL with lipoprotein lipase but does not interfere with the uptake of αₙ-macroglobulin or tissue plasminogen activator, all known to be ligands for the LRP. The precise mechanisms whereby lactoferrin inhibits remnant binding and interaction, however, remain to be determined and are the subject of this study.

The apoE-enriched β-VLDL display enhanced binding and uptake by a variety of cells in culture, including fibroblasts lacking LDL receptors, compared with β-VLDL without the addition of exogenous apoE. The postulated mechanism for the enhanced binding and uptake includes at least two steps: initial binding of the apoE-enriched remnants to heparan sulfate proteoglycans (HSPG) followed by uptake presumably mediated either by the LRP after transfer of the remnants to this receptor or by an HSPG-LRP complex. This model is supported by evidence showing that 80% or more of the enhanced binding of β-VLDL+human apoE3 can be abolished by heparinase treatment of various cells in culture. In addition, wild-type Chinese hamster ovary (CHO) cells display enhanced binding of these apoE-enriched remnants; however, mutant CHO cells (pgsD-677) lacking HSPG do not demonstrate increased binding or uptake. The low level of basal binding of β-VLDL+apoE3 to the mutant CHO cells is mediated by LDL receptors. The wild-type and mutant CHO cells display similar levels of functional LRP, as demonstrated by the binding of 125I-αₙ-macroglobulin to these cells. Thus, in the absence of cell-surface HSPG, apoE-enriched remnants do not appear to bind to a significant extent directly to the LRP.
Therefore, consideration was given to the possibility that lactoferrin inhibits remnant lipoprotein binding and uptake by interacting with cell-surface HSPG. Lactoferrin, a 76-kd glycoprotein, contains a cluster of four arginine residues that resemble the receptor-binding domain of apoE. The present study demonstrated that lactoferrin binds to HSPG as well as to the LDL receptor and suggests that the inhibition of plasma clearance of chylomicron remnants may be due to interference with the initial phase of remnant clearance, i.e., the sequestration of apoE-enriched remnants in the space of Disse by HSPG interaction.

## Methods

Rabbit β-VLDL were isolated from the plasma of New Zealand White rabbits that had been fed a high-fat, high-cholesterol diet for 4 days as described by Kowal et al. The lipoproteins were centrifuged twice at 1,000 g/mL and iodinated by the method of Bilheimer et al. Free iodine was removed by using a PD-10 column (Pharmacia LKB, Uppsala, Sweden). The 39-kd protein is also referred to as the receptor-related protein. Lactoferrin and the 39-kd fusion protein were iodinated with IOODO-GEND (Pierce Chemical Co, Rockford, IL). The 39-kd protein was isolated by using a 0.2 to 1.0 mol/L NaCl gradient. Therefore, consideration was given to the possibility that lactoferrin inhibits remnant lipoprotein binding and uptake by interacting with cell-surface HSPG. Lactoferrin, a 76-kd glycoprotein, contains a cluster of four arginine residues that resemble the receptor-binding domain of apoE. The present study demonstrated that lactoferrin binds to HSPG as well as to the LDL receptor and suggests that the inhibition of plasma clearance of chylomicron remnants may be due to interference with the initial phase of remnant clearance, i.e., the sequestration of apoE-enriched remnants in the space of Disse by HSPG interaction.

### Results

Lactoferrin competes with 125I-β-VLDL-human apoE3 for binding to HepG2 cells (Fig 1A). Approximately 75% of the binding was inhibited at a concentration of 40 μg/mL lactoferrin, and 90% was inhibited at 100 μg/mL lactoferrin. Likewise, lactoferrin inhibited the binding of 125I-β-VLDL+apoE to FH fibroblasts (=85% at 40 μg/mL) (Fig 1B). These results are consistent with those of Willnow et al, who have demonstrated that lactoferrin is an excellent competitor for apoE-enriched β-VLDL. On the other hand, lactoferrin (100 μg/mL) did not decrease the binding of 125I-β-VLDL without added apoE to HepG2 cells. We have shown that the enhanced binding of apoE3-enriched β-VLDL is primarily mediated by cell-surface HSPG, that heparanase treatment of cultured cells inhibits the binding of apoE-enriched β-VLDL by more than 80%, and that mutant CHO cells (pgsD-677) lacking HSPG do not display an enhanced binding of apoE-enriched β-VLDL. Therefore, consideration was given to the possibility that lactoferrin competes with apoE-enriched β-VLDL for binding by interacting with HSPG and presumably also with the LRP.

To examine in more detail the inhibition of apoE-enriched β-VLDL binding by lactoferrin, competitive binding studies (4°C) were performed in wild-type CHO cells and mutant CHO cells lacking HSPG by using 125I-β-VLDL with or without added apoE versus increasing concentrations of unlabeled lactoferrin. Lacto-
ferrin inhibited $^{125}$I-β-VLDL+apoE binding to wild-type CHO cells by >80% but had much less effect on the binding of $^{125}$I-β-VLDL without added apoE (<10% to 15% inhibition) (Fig 2A). On the other hand, lactoferrin was not an effective competitor against either β-VLDL or apoE-enriched β-VLDL in the mutant CHO cells lacking HSPG (Fig 2B). We have shown that the enhanced binding of apoE-enriched β-VLDL to wild-type CHO cells is largely inhibited by heparinase treatment, which removes the HSPG, and that the binding to mutant CHO cells is primarily mediated by LDL receptors, even though these cells possess functional LRP.18 These results suggest that lactoferrin competes with apoE-enriched β-VLDL in the wild-type CHO cells by interfering with the binding of the remnants to the HSPG; however, in the cells lacking HSPG, lactoferrin does not significantly compete for the low level of enhanced binding of the apoE-enriched β-VLDL.

Direct binding studies of $^{125}$I-lactoferrin were undertaken in HepG2 and CHO cells to establish the importance of HSPG binding. Approximately 45% to 60% of the lactoferrin binding to the HepG2 and wild-type CHO cells could be inhibited by heparinase treatment of the cells (3 U/mL at 37°C for 2 hours), suggesting that this level of lactoferrin binding was to HSPG (Fig 3). In addition, the mutant CHO cells (pgsD-677) lacking HSPG bound much less $^{125}$I-lactoferrin than did the wild-type CHO cells, and the residual binding to these cells was not affected by heparinase treatment (Fig 3). The binding in the absence of HSPG may represent the amount of lactoferrin bound directly to the LRP.

Since it appeared that in the HepG2 and CHO cells ~50% of the lactoferrin binding was to HSPG and since apoE-enriched β-VLDL binds primarily to cell-surface HSPG, it was of interest to determine whether increasing concentrations of unlabeled β-VLDL or β-VLDL enriched in apoE could compete with $^{125}$I-lactoferrin for 4°C binding. About 40% of the $^{125}$I-lactoferrin could be displaced from HepG2 cells at the highest concentration of apoE-enriched β-VLDL used in these studies (Fig 4A), but β-VLDL without added apoE, which does not to bind to HSPG,8 did not inhibit the binding of the $^{125}$I-lactoferrin. Similar to results obtained with the HepG2 cells, apoE-enriched β-VLDL displaced about 40% to 45% of the $^{125}$I-lactoferrin from the wild-type CHO cells at a concentration of 200 μg protein/mL (Fig 4B). However, even though the pgsD-677 mutant CHO cells retained significant binding of $^{125}$I-lactoferrin (presumably interacting with the LRP; see Fig 3), the β-VLDL enriched in apoE did not compete with the lactoferrin for binding to these cells. This finding fur-
These results are similar to those described for the binding of lactoferrin to the CHO cells (Fig 3). Therefore, a deficiency of HSPG created by heparinase treatment or by a mutation leading to an absence of HSPG inhibits lactoferrin uptake and degradation by 45% to 60% (Table).

The 39-kd protein associated with the LRP has been shown to compete with all the ligands for the LRP5.6,19-27 and to inhibit the degradation of lactoferrin by cultured cells.5 Therefore, as shown in Fig 7 and the Table, the 39-kd protein was used to determine its effect on 125I-lactoferrin degradation in the CHO cells. The degradation was decreased =60% to 65% in the wild-type CHO cells by the addition of 60 μg of the 39-kd protein/mL. The combination of heparinase treatment and the 39-kd protein further inhibited the degradation of the lactoferrin in the wild-type CHO cells by =85%. In additional studies using higher concentrations of the 39-kd protein alone (up to 100 μg/mL), it was possible to decrease the degradation of 125I-lactoferrin by =70% in the wild-type CHO cells. Furthermore, the combination of heparinase treatment and the addition of 100 μg/mL of the 39-kd protein reduced lactoferrin degradation by 92%.

In the mutant CHO cells lacking HSPG there was much less degradation of the 125I-lactoferrin (=40% compared with the wild-type CHO cells), and the
addition of the 39-kd protein inhibited this degradation by \( \approx 60\% \). As would be expected in the cells lacking HSPG, the combination of heparinase treatment and the 39-kd protein did not further decrease degradation of lactoferrin over the results with the 39-kd protein alone. Therefore, these data and results from other studies (Table) suggest that the 39-kd protein competed with the LRP-mediated uptake of the lactoferrin in the pgsD-677 mutant CHO cells since no HSPG was present to mediate binding and uptake. The residual binding of the lactoferrin may reflect incomplete competition by the 39-kd protein or the presence of an additional site on the cell surface that is responsible for interacting with the lactoferrin. However, the marked decrease in lactoferrin uptake and degradation by the 39-kd protein in the wild-type CHO cells suggested that the 39-kd protein inhibited interaction of the lactoferrin with both HSPG and the LRP.

To determine whether the \( ^{125}\text{I}-39\text{-kd protein} \) bound directly to cell-surface HSPG, its binding to wild-type and mutant CHO cells was investigated with and without heparinase treatment of the cells. Heparinase treatment of wild-type CHO cells caused a 40% decrease in the binding of the \( ^{125}\text{I}-39\text{-kd protein} \); however, the mutant CHO cells bound significantly less \( ^{125}\text{I}-39\text{-kd protein} \) compared with the wild-type CHO cells, and heparinase treatment had no effect on this level of binding (Fig 8). From these studies we conclude that the 39-kd protein binds to HSPG and that it could inhibit lactoferrin binding by interacting with both HSPG and the LRP.

**Discussion**

The inhibition of plasma clearance of apoE-enriched remnant lipoproteins by lactoferrin could be mediated by its ability to bind to cell-surface HSPG as well as by its direct interaction with the LRP. Remnant clearance and uptake by the liver appear to involve several steps. First, the initial, rapid clearance of remnants from the plasma involves a sequestration of these lipoproteins within the space of Disse, presumably through binding to HSPG. ApoE secreted by the hepatocytes would be available to enrich the remnants with this apolipoprotein and to mediate the enhanced binding to HSPG. Second, the remnants may be further processed by lipases in the space of Disse that are also likely bound to HSPG (hepatic lipase) or carried into this space in complex with the lipoprotein particles (lipoprotein lipase). Lipoprotein lipase\(^5,23,28\) and hepatic lipase\(^26\) enhance the binding of remnant lipoproteins to HSPG or the LRP. The final step involves the uptake of the remnants by the LDL receptor\(^29\) and the LRP.\(^1,13,32\) As we have speculated,\(^8,9\) the HSPG-bound remnant lipoproteins may be transferred to the LRP, or the HSPG and the LRP may function as a complex to initiate the internalization of the particles.

Lactoferrin was shown to bind to the rat liver LRP, confirming a previous observation,\(^3\) and to matrix HSPG isolated from the rat liver. Lactoferrin avidly competed with apoE-enriched \( \beta \)-VLDL in HepG2 and CHO cells. In addition, \( ^{125}\text{I}-\text{lactoferrin} \) bound directly to the cells, and \( \approx 50\% \) of the binding was inhibited by...
Effect of Heparinase Treatment and 39-kd Protein on Degradation of $^{125}$I-Lactoferrin in Wild-Type CHO Cells and CHO Cells Lacking HSPG

<table>
<thead>
<tr>
<th>$^{125}$I-Lactoferrin, μg/mL</th>
<th>Untreated (Control), ng/mg cell protein</th>
<th>Heparinase, ng/mg cell protein</th>
<th>39-kd Protein,† ng/mg cell protein</th>
<th>Heparinase + 39-kd Protein,‡ ng/mg cell protein</th>
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<tr>
<td>Wild-type CHO cells</td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>530±103</td>
<td>292±84</td>
<td>176±28</td>
<td>59±4</td>
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<tr>
<td>30</td>
<td>1403±301</td>
<td>896±90</td>
<td>487±154</td>
<td>245±119</td>
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<tr>
<td>CHO cells lacking HSPG</td>
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<tr>
<td>5</td>
<td>166±24</td>
<td>173±29</td>
<td>68±23</td>
<td>61±13</td>
</tr>
<tr>
<td>30</td>
<td>661±199</td>
<td>600±224</td>
<td>231±54</td>
<td>275±21</td>
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CHO indicates Chinese hamster ovary; HSPG, heparan sulfate proteoglycan. Results are presented as nanograms $^{125}$I-lactoferrin degraded per milligram of cell protein in 5 hours (mean±SD). Two separate experiments, each in duplicate, were performed.

* Heparinase treatment was 3 U/mL at 37°C for 6 hours.
† Heparinase treatment (3 U/mL) and added 39-kd protein (60 μg/mL).
‡ Heparinase treatment (3 U/mL) and added 39-kd protein (60 μg/mL).

Treatment of the cells with heparinase, which hydrolyzes the cell-surface HSPG. Likewise, mutant CHO cells lacking HSPG bound only about half as much $^{125}$I-lactoferrin as wild-type CHO cells. Thus, lactoferrin binding and competition are mediated to a significant extent by HSPG interaction.

The 39-kd protein, which is known to bind to the LRP and to inhibit the interaction of various ligands to the LRP, inhibited the uptake of $^{125}$I-lactoferrin by CHO cells (±60%), and the combination of heparinase treatment and the addition of the 39-kd protein resulted in a further marked inhibition of uptake that reflected an interference of lactoferrin binding to both HSPG and the LRP. However, using the 39-kd protein as a competitor, it was not possible to estimate precisely the amount of HSPG versus LRP binding because the 39-kd protein was also shown to bind to the cell-surface HSPG. Heparinase treatment decreased the binding of the $^{125}$I-39-kd protein by ±40% in wild-type CHO cells, and the mutant CHO cells lacking HSPG bound less than half as much of the $^{125}$I-39-kd protein as did the wild-type CHO cells. Wild-type and mutant CHO cells express equal levels of LRP activity.

The 39-kd protein binds to heparin, and Warshawsky et al.17 have further characterized a specific fragment of the 39-kd protein that mediates its interaction with heparin. In addition, Chappell et al.34 have demonstrated that the 39-kd protein competes with lipoprotein lipase binding to fibroblasts lacking LDL receptors. Most of the lipoprotein lipase was not bound to the LRP, and thus Chappell et al suggest that the competition occurred via the displacement of lipoprotein lipase from cell-surface HSPG by the 39-kd protein. On the other hand, Vassiliou and Stanley35 report that the 39-kd protein binds to two distinct sites on human fibroblasts with high and low binding affinities. However, they did not find significant binding of the 39-kd protein to cell-surface HSPG. Heparinase or sodium chlorate treatment did not significantly decrease the binding of the 39-kd protein to fibroblasts. These results differ from ours using CHO cells. The reason for this apparent discrepancy may relate to the specific cells studied (fibroblasts versus CHO cells) and may be explained on the basis of the specific types of HSPG displayed on the surface of the various cell types. This remains to be determined.

These studies further substantiate the conclusion that the enhanced binding of apoE-enriched β-VLDL to cells in culture is mediated primarily through an interaction with cell-surface HSPG, with very little direct binding to the LRP. Iodinated lactoferrin appeared to bind directly to the LRP of mutant CHO cells lacking HSPG and could not be displaced even with high concentrations of apoE-enriched β-VLDL. This suggests that apoE-enriched β-VLDL do not interact significantly with the LRP on the surface of cells in the absence of HSPG (although apoE-enriched β-VLDL clearly bind directly to the LRP on ligand blots).6 Alternatively, it is possible that lactoferrin has a higher affinity than do apoE-enriched β-VLDL for the LRP or that lactoferrin binds to a different site on the LRP of the mutant CHO cells. However, this latter suggestion is inconsistent with previous results demonstrating that lactoferrin inhibits cholesteryl ester biosynthesis induced by apoE-enriched β-VLDL in fibroblasts lacking LDL receptors, reflecting an inhibition of uptake of the lipoproteins by the LRP. It appears that cell-surface HSPG may be required to allow the transfer of apoE-enriched remnants to the LRP for internalization or that HSPG and the LRP form a complex that is necessary for uptake of the remnants. Interaction of apoE-enriched remnants with HSPG may facilitate
their access to the LRP and allow remnant binding to the LRP to occur on the cell surface. On the other hand, lactoferrin appears to interact directly with HSPG and the LRP, and thus can inhibit the metabolism of remnant lipoproteins by preventing the sequestration phase of clearance (HSPG binding) or the subsequent transfer of the remnants to the LRP.

Acknowledgments

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Lactoferrin binding to heparan sulfate proteoglycans and the LDL receptor-related protein. Further evidence supporting the importance of direct binding of remnant lipoproteins to HSPG.

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