Lactosaminated Fab Fragments Specific for Low Density Lipoproteins/Hepatocyte Targeting and Hypolipoproteinemic Activity

Franco Bernini, David P. Via, Thomas M.A. Bocan, Antonio M. Gotto Jr., and Louis C. Smith

We have previously reported that Fab fragments of IgGs modified by lactosamination (lac-Fab) can direct macromolecules, including low density lipoproteins (LDL), to the liver. In the present paper we demonstrate that lac-Fab that is specific for LDL is an effective and selective hypolipoprotein agent. A plasma pool of about 60 mg/dl of apoprotein B (apo B) was induced in rats by bolus injection of human LDL (hLDL), which increased the cholesterol value to about 150 mg/dl. Three hours after injection of the highest dose of lac-Fab, the total cholesterol decreased to 80 mg/dl, compared to 120 mg/dl in control animals. Studies conducted with 131I-tyramine-cellobiose-labeled LDL indicated that the liver was the only organ in which lac-Fab increased LDL uptake and degradation. The effect of lac-Fab was dose-dependent. With amounts of lac-Fab between 13 to 42 mg/kg body weight, the amount of hLDL cleared through the lac-Fab mechanism ranged from 30% to 70% of the initial pool. Analysis of the plasma lipoprotein subfractions revealed that high density lipoprotein levels were not affected. Histologic examination of liver sections after sequential injection of fluorescently labeled hLDL and lac-Fab indicated specific uptake in the hepatocytes when compared to control sections obtained from animals injected with Dil-LDL alone. The uptake of fluorescent LDL induced by lac-Fab was completely prevented by a co-injection of an excess of asialofetuin. We conclude that lac-Fab that is specific for LDL is a selective hypolipoproteinemic agent and a specific carrier to the hepatocytes. (Arteriosclerosis 8:825–831, November/December 1988)

Specific hepatic receptors for apolipoproteins are fundamentally important for the normal metabolism of the lipoproteins and lipids that they transport. It is well-documented that the plasma level of lipids becomes abnormal, with increased risk of atherosclerosis, when the interaction of the lipoprotein apoprotein components with their receptor is impaired through defects of either the receptor or the ligand. Internalization and degradation of these ligands in the lysosomes involve processes that are analogous to those described for low density lipoproteins (LDL).

We have previously reported that, after lactosamination, IgG Fab fragments retain their antigen specificity and are recognized by the asialoglycoprotein receptor of the liver. This type of reagent was able to direct macromolecules, including LDL, specifically to the liver, thereby promoting their plasma clearance. Therefore we proposed that the lac-Fab could be used to provide specific hepatic clearance for both exogenous and circulating macromolecules when the normal specific metabolism is absent or not sufficient for the requirements of normal homeostasis.

An agent designed to reduce hyperlipidemia has the following desirable features: The agent should remove from circulation sufficient amounts of the atherogenic lipoproteins such as LDL without affecting other lipoproteins such as HDL that are negatively correlated with cardiovascular diseases. Secondly, the hypcholesterolemic mechanism should involve the liver, the only organ that metabolizes cholesterol for excretion.

In this paper we demonstrate that lac-Fab has the pharmacologic properties of an effective and selective hypolipoproteinemic agent. This reagent makes possible an interesting experimental technique to selectively change the plasma levels of different lipoprotein fractions. We also report morphological evidence that lac-Fab and its complexes are cleared by the liver parenchymal cells.

Methods

Lac-Fab Preparation

The lac-Fab was prepared as previously described. In brief, the Fab fragment was prepared by papain digestion of an affinity-purified sheep IgG specific for human LDL (hLDL).
The purified Fab was then derivatized by reductive lactosamination in the presence of sodium cyanoborohydride.

**Low Density Lipoprotein Preparation and Radioactive Labeling**

LDL (1.019<d<1.063) was separated from normal plasma, which was obtained from the Methodist Hospital Blood Bank, by preparative ultracentrifugation in KBr. This was dialyzed against phosphate-buffered saline. A portion of the LDL was iodinated by the iodine monochloride method. More than 99% of the LDL radioactivity was insoluble in 10% trichloroacetic acid. LDL labeled with \(^{131}I\)-tyramine-cellulbiose-adduct (\(^{131}I\)-TC-LDL) were kindly provided by Ray C. Pittman, University of California, San Diego.

**Low Density Lipoprotein Fluorescent Labeling**

LDL were labeled with \(1,1\)-diiodoacetetyl-3,3,3,3'-tetramethylindocarbocyanine perchlorate as previously described.14,15

**Plasma Clearance and Organ Uptake Studies**

Male Sprague-Dawley rats (n=40) were obtained from Harlan (Houston, TX) and housed in approved institutional vivarium facilities. Fasting male Sprague-Dawley rats weighing 150 to 250 g were fitted with femoral vein catheters under ether anesthesia. After recovery from anesthesia, the rats received 23 mg/kg body weight of LDL protein containing about 1.5 to 5x10^6 cpm of radioactive LDL in a single bolus. Ten minutes later they received lac-Fab or an equal volume of buffer. The volume injected was always less than 10% of the plasma volume, which was calculated as 4.2% of the body weight. Samples were injected and blood was sampled through the femoral vein catheter as described.6 For analysis of organ uptake, a similar protocol was observed, except that \(^{131}I\)-TC-LDL was substituted for the \(^{125}I\)-LDL. Animals were anesthetized with 50 mg/kg sodium pentobarbital 60 minutes after the fluorescent LDL injection, then they were exsanguinated and perfused with 60 ml of saline via the femoral catheter. The liver was removed, washed in 0.9% saline, and flash-frozen in n-hexane chilled by a CO2-ethanol slurry. The samples were stored at -20°C until sectioned. The 10 μm sections were cut on an AO Histostat and flash-dried onto acid-alcohol cleaned, polylysine-coated slides. The slides were mounted in 90% glycerol in phosphate-buffered saline, and the locations of the fluorescent markers were determined by fluorescence microscopy by using a standard rhodamine filter package.

**Results**

To demonstrate that lac-Fab could be effective in reducing the plasma concentration of LDL, the amount of cholesterol circulating in the rat was increased by a bolus injection of hLDL and \(^{125}I\)-hLDL, equivalent to 50 to 60 mg/dl of human apo B. Consequently, the plasma total cholesterol was increased to about 120 to 150 mg/dl, of which 50 to 90 mg/dl was contained in hLDL. The serum decay of \(^{125}I\)-hLDL and hLDL cholesterol in the animals that received only hLDL was slow and followed a time course typical of that reported by others17,18,19 (Figure 1). The injection of a dose, 42 mg/kg body weight of lac-Fab 1.8-fold larger than the hLDL pool dramatically increased the disappearance rate of both radioactivity and cholesterol from serum (Figure 1). Three hours after the injection of lac-Fab, about 80% of the initial hLDL pool had disappeared from circulation, compared to about 25% in the rats receiving hLDL but not lac-Fab. A similar result was obtained by evaluating changes in hLDL-associated cho-
LESTEROL. In some, but not all, the animals the serum cholesterol concentration increased slightly after 3 hours. After 6 hours, the total plasma cholesterol concentration was reduced about 24% in control animals and 40% in animals receiving the lac-Fab, corresponding to a decrease of hLDL cholesterol from 74.2 ± 20.0 to 47.7 ± 9.3, and 74.0 ± 23.5 to 15.1 ± 10.1 mg/dl, respectively.

To evaluate the site of uptake and degradation of the lac-Fab/LDL immunocomplex, animals were treated exactly as described above except that 125I-TC-LDL was substituted for 125I-LDL. As described by Pittman and co-workers,20 this label is trapped intracellularly after uptake or catabolism of the lipoproteins. This provides an accurate determination of the tissue sites of uptake and degradation. The data in Table 1 show that lac-Fab reduced the cholesterol levels by about 24% in control animals and 35% in animals receiving the lac-Fab, corresponding to a decrease of hLDL cholesterol from 74.2 ± 20.0 to 47.7 ± 9.3, and 74.0 ± 23.5 to 15.1 ± 10.1 mg/dl, respectively.

The ability of lac-Fab to reduce the cholesterol levels induced by the bolus injection of hLDL appears to be specific for the low density range of lipoproteins. The injection of 42 mg of lac-Fab per kilogram of body weight had no effect on cholesterol concentration in the density range of high density lipoprotein (HDL) (Table 2). The specificity of the hypolipoproteinemic activity of lac-Fab was also confirmed by zonal ultracentrifugation of collected plasma (Figure 2B). As expected, the bolus injection of HDL produced a complete disappearance of this peak without affecting LDL (Figure 2C) and restored the lipoprotein distribution profile in the serum to that obtained from normal control rats (Figure 2A).

The amount of the 125I-hLDL cleared from serum depended on the dose of lac-Fab administered to the animals (Figure 3). Groups of two animals each received a bolus injection of 23 mg/kg body weight of hLDL and a trace amount of 125I-hLDL. After 10 minutes, they received an injection of 0 to 42 mg of lac-Fab per kilogram of body weight. These data, which are summarized in Table 3, show that the amount of hLDL catabolized through the lac-Fab mechanism was dependent on the dose of lac-Fab administered, resulting in a maximum clearance of 73% of the initial pool.

At the highest dose of lac-Fab, it was calculated that in the first hour after injection, at least 6.9 mg/kg of human apo B could be cleared. A comparable value, 6.5 mg/kg, was obtained when lac-Fab was incubated for 1 hour with hLDL before injection into the animal. In this experiment, the molar ratio of lac-Fab to hLDL in the pre-injection incubation was 13, which was found to be the maximum amount of lac-Fab that could be associated with hLDL.6
Table 3. Percentage of $^{125}$I-Human Low Density Lipoprotein Cleared by Lac-Fab as Function of Dose

<table>
<thead>
<tr>
<th>Lac-Fab injected (mg/kg body weight)</th>
<th>Lac-Fab/hLDL (wt/wt ratio)</th>
<th>Amount of hLDL cleared (% of initial pool)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>0.6</td>
<td>32.2</td>
</tr>
<tr>
<td>21</td>
<td>0.9</td>
<td>39.8</td>
</tr>
<tr>
<td>28</td>
<td>1.2</td>
<td>57.1</td>
</tr>
<tr>
<td>42</td>
<td>1.8</td>
<td>73.1</td>
</tr>
</tbody>
</table>

Values were obtained by extrapolation from the intercept on the y axis of the linear component of the last portion of each curve in Figure 3. The correlation coefficients for the best fit were 0.99 except for the last value, for which $r=0.94$.

Figure 2. The effect of lac-Fab on low density lipoprotein (LDL) and high density lipoprotein plasma content. Left panels, $d<1.063$. Right panels, $1.063<d<1.21$. A. Control. B. hLDL alone. C. hLDL and lac-Fab. Experimental conditions are described in Table 2. The plasma lipoprotein distribution was determined by zonal ultracentrifugation as described in the Methods section.

It has been reported that another class of receptors, which are present on liver macrophages, are responsible for clearance of modified LDL with exposed galactose residues. It was therefore important to determine which cells in the liver were clearing the hLDMac-Fab complexes.

Frozen sections of liver prepared 60 minutes after the injection of lac-Fab in rats pretreated with a bolus of Dil-labeled-LDL were examined by fluorescence microscopy. The fluorescence was localized in the parenchyma of the liver (Figure 4B). No appreciable fluorescence was seen in the nonparenchymal cells lining the sinusoids. To confirm that the fluorescence observed in the hepatocytes was mediated by lac-Fab interaction with LDL, these sections were compared with those from animals injected with Dil-LDL alone, which is poorly taken up by the rat liver. In the liver of animals receiving Dil-LDL alone, little fluorescence was detected (Figure 4D). Finally, to demonstrate further that the uptake of Dil-LDL induced by lac-Fab could be mediated by galactose receptors on the hepatocytes, animals receiving lac-Fab were also treated with an excess of asialofetuin. This prevents the uptake of galactose-lipoproteins only by the hepatocytes and not by the nonparenchymal cells. Figure 4F shows that in this condition no fluorescence was detected in the liver sections. The faint fluorescence in Figures 4D and 4F is probably due to the autofluorescence of stromal support elements of the liver.

Discussion

The catabolism of hLDL in rat is mediated by a low affinity nonspecific uptake. This observation is supported by several kinds of experimental evidence. Modification of LDL with 1,2-cyclohexanedione does not change the catabolic rate in the rat, nor is the rate of clearance affected by the amount of LDL injected. In our experiments, the serum decay of the hLDL in rats for radioactivity and cholesterol had a half-life of about 7.5 hours, in agreement with similar results reported by others. Using this experimental model, we have shown that lac-Fab can rapidly increase the plasma clearance of physiologically relevant amounts of hLDL. Such an effect appears to be due exclusively to an increased lac-Fab-induced uptake of LDL by the liver without any significant increase in the uptake by the other organs examined. About 50% of the pool of the injected hLDL disappeared 1 hour after the injection of 42 mg/kg of lac-Fab as compared to about 15% in the controls, based on both $^{125}$I radioactivity and cholesterol content of the serum. In previous experiments with trace amounts of radioactive LDL and only 3 mg of lac-Fab per kilogram of body weight, 50% of the initial radioactivity was cleared in about 10 minutes. This observation illustrates the spec-
Figure 4. The effect of lac-Fab on hepatic localization of fluorescent lipoproteins. Rats were injected intravenously with Dil-LDL and lac-Fab (A,B), Dil-LDL alone (C,D), or Dil-LDL with lac-Fab and asialofetuin (E,F). Phase contrast images are shown in the left column and the corresponding fluorescence image, in the right column. Frozen sections of rat liver were prepared and examined as described in the Methods section. H=hepatocyte, S=sinusoid, CV=central vein. \( \times 368 \).

Specificity and saturability of the mechanism by which the hypolipoproteinemic activity of the lac-Fab is achieved.

Pre-incubation of the LDL with lac-Fab before injection in the animals gave a value for the rate of clearance of the LDL comparable to that obtained by injecting the lac-Fab separately. This result indicates that the rate of association of lac-Fab with circulating lipoprotein is rapid and that negligible amounts of lac-Fab are cleared from the circulation before its association with LDL.

Since the Fab fragment used in our experiments had little, if any, affinity for the rat lipoproteins, especially HDL, we expected a high degree of selectivity for the hypocholesterolemic and hypolipoproteinemic activity of the lac-Fab. The validity of this expectation has been shown by analysis of the lipoprotein and cholesterol content in the plasma after ultracentrifugation. Only cholesterol and apoproteins in the density range less than 1.063 were cleared from circulation. Thus, the hypolipoproteinemic effect of lac-Fab is linked to the specificity of the IgG from which the Fab fragment was obtained. It seems reasonable to assume that lac-Fabs with different antigenic specificity for apoproteins can...
be used to selectively modulate the plasma levels of specific apoproteins or lipoproteins.

Liver macrophages reportedly can bind particles, including modified LDL, that bear galactose terminal residues.\textsuperscript{21,26,27} Similar binding properties have also been reported for peritoneal macrophages.\textsuperscript{28} Accumulation of cholesterol in macrophages and formation of foam cells is considered to be one of the major events involved in the pathogenesis of atherosclerosis.\textsuperscript{29} It was, therefore, important to assess the target specificity of the lac-Fab. We previously reported that the ability of lac-Fab to induce uptake of LDL by both the hepatocytes in culture and by the liver in vivo was completely antagonized by an excess of asialofetuin.\textsuperscript{9} Asialofetuin inhibits the binding of lipoproteins that bear terminal galactose residues to the galactose receptor of the hepatocytes but not of the nonparenchymal cells.\textsuperscript{21,23} The specificity of lac-Fab for hepatocytes was anticipated by the asialofetuin competition studies. In the present paper, more direct evidence has been provided. The studies with fluorescent Dil-LDL and lac-Fab gave visual evidence of the specificity of uptake of lac-Fab for the hepatocytes. If any fluorescence was present in the nonparenchymal cells, it would appear as intense punctate fluorescence within the sinusoids, as demonstrated for Dil-AC-LDL by Pitas et al.\textsuperscript{29} Figure 4B shows no such fluorescent pattern with lac-Fab/Dil-LDL, but only an intense uniform fluorescence within the parenchymal cells and no fluorescence within the sinusoids. This observation is supported by the control studies with Dil-LDL alone, which is poorly taken up by the liver.\textsuperscript{22} Moreover, an excess of asialofetuin completely prevented Dil-LDL uptake in the liver induced by lac-Fab, indicating that asialofetuin can fully compete with the lac-Fab/Dil-LDL immunocomplex for the receptors on the hepatocytes and that these receptors are entirely responsible for the lacFab/Dil-LDL uptake. Taken together, our data support the view that lac-Fab is a specific carrier to the asialoglycoprotein or galactose receptor of the hepatocytes.

The possibility of using LDL as a carrier of lipophilic substances and drugs has been reviewed.\textsuperscript{30} The lac-Fab/LDL complex appears to be a promising means for transport of pharmacologically active molecules that are too insoluble for other routes of administration. It would be interesting, for example, to evaluate the effectiveness and specificity of lipophilic inhibitors of cholesterol synthesis incorporated into the lipid core of LDL that were delivered specifically to the liver. This strategy could increase their efficacy as liver-specific agents and reduce the possible side effects of systemic administration of such agents.

Lac-Fab is a new kind of reagent that we consider to be a useful experimental tool. It will need to be improved or modified before it can be considered as a therapeutic agent in humans due to the risk of immune complex deposition associated with any antibody reagent. However, the data in this paper clearly demonstrate that lac-Fab itself is a highly selective reagent with many possible experimental applications for in vivo lipoprotein metabolism.

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References


Index Terms: Fab • LDL • galactose receptor • hepatocyte • hypolipoprotein agent
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