Macrophage-Conditioned Medium and β-VLDLs Enhance Cholesterol Esterification in SMCs and HSFs by LDL Receptor–Mediated and Other Pathways

O. Stein, Y. Dabach, M. Ben-Nairn, G. Hollander, Y. Stein

Thioglycolate-elicited mouse peritoneal macrophages were incubated for 24 hours in serum-free Dulbecco-Vogt medium containing 0.5% fatty acid–poor bovine serum albumin. This conditioned medium, designated MP medium, was used for experiments with bovine aortic smooth muscle cells (SMCs) or human skin fibroblasts (HSFs). Dulbecco-Vogt medium of the same albumin content but without macrophages served as a control medium. In SMCs labeled from plating with [3H] cholesterol and incubated with hypercholesterolemic rabbit β-very-low-density lipoprotein (β-VLDL) in Dulbecco-Vogt medium for 24 hours, there was an increase in cellular [3H] cholesteryl ester (CE) content compared with cells incubated without lipoprotein. When MP medium was used for the incubation of SMCs with β-VLDL, cellular [3H] cholesteryl ester content increased threefold compared with cells incubated with Dulbecco-Vogt medium. A smaller increase in cholesterol esterification in the presence of MP medium was also encountered with low-density lipoprotein (LDL). The MP medium-induced increase in [3H] cholesteryl esterification was not evident up to 6 hours of incubation. Similar results were also obtained with HSFs. The increase in [3H] cholesteryl esterification with MP medium in the presence of β-VLDL was also elicited in cells obtained from LDL receptor-negative donors with familial hypercholesterolemia (FH-HSF), even though in these cells significantly less [3H] cholesteryl ester was formed in the presence of 0-VLDL. MP medium contains numerous agents that could be responsible for the increase in cellular [3H] cholesteryl ester induced by lipoproteins. The first considered was lipoprotein lipase, but lack of inhibition of the MP medium effect by antiserum to lipoprotein lipase did not support this possibility. Recombinant apoprotein E, when added to β-VLDL and Dulbecco-Vogt medium, failed to mimic the MP effect, which argued against apoprotein E's being the "active" agent. Since the increase in cellular [3H] cholesteryl ester in the presence of β-VLDL and MP medium was more prominent in normal HSFs and SMCs than in FH-HSFs, it appears that although the LDL receptor may play a role, it is not absolutely necessary. The exact mode by which MP medium enhances cellular [3H] cholesteryl ester formation in the presence of β-VLDL has not been elucidated, but it is important to conclude that this may occur through multiple pathways involving both the uptake of the whole particle and preferential uptake of the lipid moiety.

Key Words • human skin fibroblasts • familial hypercholesterolemia • cholesteryl ester • LDL • LDL receptor–related protein • lipoprotein lipase • apoprotein E • proteoglycan • lactoferrin

The macrophage appears to play a pivotal role in the incipient stage of atherosclerosis. After a few weeks on a cholesterol-rich diet, macrophages in the aorta of monkeys and rabbits accumulate cholesteryl ester (CE), are transformed into foam cells, and give rise to the fatty streak. Later the smooth muscle cells (SMCs) start to proliferate, acquire lipid, and are also transformed into foam cells. The enigma that still eludes us is the mechanism by which CE accumulates in SMCs. Is it a receptor- or nonreceptor-mediated event? Three candidate receptors could play a role in the accumulation of CE in the SMC. The first is the classic low-density lipoprotein (LDL) receptor that recognizes apoprotein (apo) B and apoE, but that may not be active in this process, as it is downregulated by incoming free cholesterol or its oxygen metabolites. The scavenger receptor, which plays a central role in transforming macrophages into foam cells, recognizes modified lipoproteins. This receptor has been identified in rabbit SMCs and fibroblasts, but it is apparently absent in human skin fibroblasts (HSFs) and SMCs of other species. The third receptor could be the recently described LDL receptor–related protein that interacts with apoE-rich lipoproteins but not with apoB, the main apoprotein of LDL. The LDL receptor–related
protein is considered to be a multifunctional receptor that also binds activated α2-macroglobulin and other ligands and is not downregulated during intracellular accumulation of cholesterol that is derived from the uptake of apoE-rich lipoproteins such as chylomicron remnants and β-very-low-density lipoprotein (β-VLDL). 3

The temporal relationship, in which recruitment of the macrophage precedes the appearance of SMC-derived foam cells, as well as the proximity of these two cell types in the atheroma, raised the possibility that CE accumulation in the SMC could be enhanced by a factor secreted by macrophages. 11 Indeed, the latter study has shown that macrophage-conditioned medium increases the LDL receptor number in quiescent SMCs. 11 In another study, macrophage-conditioned medium was shown to increase the number of LDL receptors in macrophages and to enhance degradation of LDL in SMCs. 12

In the present study, we showed that lipoprotein-free medium conditioned with macrophages induced cholesterol esterification in SMCs exposed to β-VLDL or LDL. It appeared that this effect was achieved by more than one pathway and could also be demonstrated in LDL receptor–negative HSFs.

Methods

Cell Cultures

Bovine SMCs were grown from explants of aortas and subcultured in Dulbecco-Vogt (DV) medium supplemented with 10% fetal bovine serum. 13 For experiments, 5 to 7 × 10^5 cells were seeded in 35-mm Falcon Petri dishes and grown for 6 to 10 days. HSFs were obtained from volunteers with informed consent and were cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum. HSFs were also obtained from patients with familial hypercholesterolemia (FH) who were LDL receptor negative (FH-HSFs). To label the SMCs and HSFs with [3H]cholesterol, the latter was added to serum contain-

Preparation of Lipoproteins

Rabbit β-VLDLs were obtained from animals fed 1% cholesterol in their diet. The sera were pooled, and the lipoproteins were separated by ultracentrifugation for 24 hours in an SW-41 rotor at 100,000g at d = 1.006 g/mL. The top was cut with the help of a tube slicer, and the lipoproteins were resuspended in 0.15 mol/L NaCl and were resedimented at the same density as above. LDLs were isolated from human plasma, separated at d = 1.019 to 1.063 g/mL for 24 hours in an SW-41 rotor, and resedimented at this density according to Havel et al. 17

Labeling of Lipoproteins

To incorporate [3H]cholesterol oleate into rabbit β-VLDL, the labeled compound (10 to 20 μCi) was placed in a test tube, the solvent was evaporated, and the dried lipid was sonicated with 2 mL saline containing 100 μL of d > 1.21 g/mL rabbit plasma. Sonication was performed four times, 30 seconds for each sonication, by using a 9-mm probe and a Braun Sonic 4000 instrument (Melsungen, FRG). The sonicated, labeled lipid, the d < 1.006 g/mL fraction of hypercholesterolemic rabbit plasma (5 to 10 mg cholesterol), and the d > 1.21 g/mL fraction of rabbit plasma, which served as a source of CE transfer protein, were incubated for 18 hours at 37°C under nitrogen. 18 The final volume was ~5 mL, and the protein concentration of the d > 1.21 g/mL fraction was 30 mg/mL. Thereafter, the lipoproteins were floated at d = 1.019 g/mL at 100,000g for 24 hours. All labeled lipoproteins were sterile filtered before use. Iodination of β-VLDL and LDL with Na [125I] was performed with the iodine monochloride method 9 as modified for the labeling of lipoproteins. 26 In LDL, more than 98% of the [125I] was in protein; in β-VLDL, 23% to 27% was in lipid. [3H]Oleic acid was bound to delipidated bovine serum albumin as previously described. 21

Preparation of Conditioned Media

For preparation of conditioned media, peritoneal macrophages or J774 cells were seeded in 60-mm Falcon dishes (10 × 10^6 cells/dish) in their respective media. After overnight incubation, the medium was removed, the cell layer was washed twice with phosphate-buffered saline (PBS), and the cells were incubated with 3 to 4 mL DV medium containing 10 mol/L N,N2-hydroxyethylpiperazine-N,N2-ethanesulfonic acid and 0.5% fatty acid–poor bovine serum albumin. This medium, which was not exposed to cells, is designated as DV medium. After 24 hours of incubation the medium was collected, centrifuged, sterile filtered, and kept at −20°C until use. This medium is designated as MP medium. In some experiments, DV medium was added to dishes containing superconfluent SMCs, collected after 24 hours, and treated as above. This medium is designated as SMC medium. In some experiments the MP medium was heated for 30 minutes at 56°C and is designated as “inactivated.”

Experimental Design

[3H]Cholesterol-labeled cells were washed briefly with PBS and then with medium containing 1% bovine serum albumin and with serum-free medium for 10 minutes each at 37°C. The cells were then incubated with either MP or DV medium and the appropriate unlabeled lipoproteins. At the end of incubation, each medium was collected, and the cells were washed twice with PBS and scraped with 1 mL 50% methanol and 2 mL 100% methanol using a Teflon policeman. After an equal volume of chloroform was added, the lipids were extracted, and the delipidated residue was used for determination of protein. 22

Nonlabeled cells used for experiments with either [3H]CE-labeled β-VLDL or [125I]-labeled β-VLDL and LDL were washed twice with PBS before the addition of labeled lipoproteins. At the end of the incubation, the medium was collected, the cell layer was washed two
times with PBS containing 0.2% albumin and once with PBS, and the cells were incubated for 60 minutes at 4°C with sodium heparin (10 mg/mL). Thereafter, the cell layer was scraped with methanol as described above. [3H]Oleic acid was added to the incubation medium for the last 2 hours of incubation.

Analytical Procedures

After extraction and purification of lipids according to Folch et al., the lipids were analyzed by thin-layer chromatography using chloroform/ethyl acetate (95:5, vol/vol) to separate [3H]cholesterol from CE. To separate [3H]cholesterol ester oleate from labeled triacylglycerol, the solvent system used was hexane/ethyl ether/acetic acid (80:20:1, vol/vol/vol).

To determine the metabolism of 125I-labeled lipoproteins, noniodide trichloroacetic acid-soluble degradation products were determined in the medium. Cellular binding after incubation at 37°C was determined as heparin-releasable label; cell-associated protein label was determined after extraction of the cells with chloroform/methanol; and lipid label was determined after evaporation of the solvents.

Lipoprotein lipase (LPL) activity in the media of macrophages was determined as previously described.

Materials

The culture media and fetal bovine serum were obtained from GIBCO (New York, NY). [7(n)-3H]Cholesterol, [3H]cholesterol ester, [9,10(n)-3H]oleic acid, and Na125I were obtained from Amersham International, UK. Bovine serum albumin and sodium heparin were obtained from Sigma, St Louis, Mo. Human recombinant apoE-3 was obtained from Biotechnology, Rehovot, Israel. LPL isolated from bovine milk was a generous gift of Dr G. Bengtsson-Olivecrona, University of Umeå, Umeå, Sweden. Antiserum to LPL was a generous gift of Dr J. Etienne, Hopital Tenon, Paris, France. All reagents were of analytical grade.

Results

To learn about a possible effect of MP medium on CE accretion in SMCs, the cells were prelabeled with [3H]free cholesterol ([3H]FC), and the appearance of the label in CE was monitored after 24 hours' exposure to nonlabeled β-VLDL or LDL (300 µg cholesterol/mL each). Data from a representative experiment (from a total of five) are shown in Fig 1; it is evident that the percent radioactivity recovered in CE in cells incubated with MP medium and β-VLDL was more than twofold higher than in those incubated with DV medium. A similar, albeit somewhat less extensive, effect was also seen with LDL (Fig 1). When SMCs were used for conditioning of the medium for 24 hours, and the medium was then added to [3H]cholesterol-labeled SMCs, its effect on CE formation in the presence of β-VLDL was not greater than that of the DV medium (Fig 1). Similar results were obtained with LDL (not shown in Fig 1). Addition of β-VLDL or LDL to DV medium increased the cellular [3H]CE compared with cells incubated without lipoproteins. This difference was even greater with HSFs, whereas the relative effects of
concentrations of \( \beta \)-very-low-density lipoprotein (\( \beta \)-VLDL) as described in Fig 1 and “Methods.” Cellular radioactivity ranged between 2.3 to 3.1 \( \times 10^5 \) dpm/dish; cellular protein was 220 µg/dish. DV indicates Dulbecco-Vogt.

DV, MP, and SMC media on cholesterol esterification were similar to those seen in SMCs (data not shown). A linear increase in \([H]\)CE in SMCs incubated with MP medium with increasing \( \beta \)-VLDL concentrations was seen up to 40 µg cholesterol/mL (Fig 2). In the presence of DV medium the increment in cellular labeled CE was evident at 40 µg \( \beta \)-VLDL cholesterol/mL and continued to increase up to 160 µg cholesterol/mL (Fig 2). When compared at 300 µg cholesterol/mL, the values were 3.6±0.1% cellular labeled CE for DV medium and 7.5±0.1% for MP medium (data not included in Fig 2).

So far, the cells used were either SMCs or normal HSFs, which express the LDL receptor. Therefore, the experiments were repeated with FH-HSFs. Using these cells we could show a similar effect of the MP medium as in the normal HSFs, albeit the magnitude of cholesterol esterification was less pronounced; thus, the MP medium effect can also be elicited in the absence of the LDL receptor (Fig 3). The concentration curve with \( \beta \)-VLDL shows a flattening between 80 and 160 µg cholesterol/mL (Fig 3).

The effect of MP medium also proved to be time dependent: at 3 and 6 hours of incubation with \( \beta \)-VLDL (100 µg cholesterol/mL) there was an increase in cellular labeled CE compared with cells incubated without the lipoprotein, but there was no difference between MP and DV media (Table 1). As in previous experiments, the difference between MP and DV media became quite apparent after 24 hours’ incubation of both HSFs and SMCs. The time dependency of the difference between MP and DV media might have been caused by an effect of the MP medium on the substrate or on the cell. To test these possibilities, the following experiments were performed: \( \beta \)-VLDL was preincubated in either DV or MP medium for 24 hours and then added to \([H]\)cholesterol-labeled SMCs for 6 hours. At that time interval, 1.6% to 2.1% of cellular \( ^{2}H \) label was found in CE irrespective of whether the medium used was MP or DV medium. The same was true for cells exposed to nonpreincubated \( \beta \)-VLDL for 6 hours, whereas after 24 hours there were 8.9% and 2.1% \([H]\)CE label when the cells were exposed to MP and DV medium, respectively. In another experiment, \([H]\)cholesterol-labeled cells were preincubated with MP or DV medium for 24 hours and then incubated for 6 hours with \( \beta \)-VLDL in either DV or MP medium. With both HSFs or FH-HSFs, no difference was seen between preincubated cells and nonpreincubated cells, irrespective of the medium used. In SMCs preincubated with MP medium, the percent \([H]\)CE in cells was two to three times higher than in control cells preincubated with DV or in nonpreincubated cells exposed to DV or MP medium for 6 hours. A 6-hour incubation of either preincubated lipoprotein or preincubated cells was cho-

**Table 1. Time Dependency of the Effect of MP Medium on \([H]\)Cholesterol Esterification in the Presence of \( \beta \)-VLDL**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Addition</th>
<th>Incubation, h</th>
<th>([H])CE, % of cellular label</th>
<th>HSFs</th>
<th>SMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>( \beta )-VLDL 3</td>
<td>2.9±0.2</td>
<td>1.1±0.07</td>
<td>1.3±0.1</td>
<td></td>
</tr>
<tr>
<td>DV</td>
<td>( \beta )-VLDL 24</td>
<td>24.5±1.6</td>
<td>7.8±0.2</td>
<td>0.6±0.06</td>
<td></td>
</tr>
<tr>
<td>MP</td>
<td>None</td>
<td>24</td>
<td>3.6±0.1</td>
<td>1.4±0.02</td>
<td></td>
</tr>
<tr>
<td>DV</td>
<td>( \beta )-VLDL 24</td>
<td>12.5±0.4</td>
<td>1.9±0.3</td>
<td>0.5±0.1</td>
<td></td>
</tr>
</tbody>
</table>

MP indicates macrophage-conditioned; VLDL, very-low-density lipoprotein; CE, cholesterol ester; HSFs, human skin fibroblasts; SMCs, smooth muscle cells; DV, Dulbecco-Vogt. The cells were labeled with \([H]\)cholesterol and washed as in Fig 1. The concentration of \( \beta \)-VLDL was 100 µg cholesterol/mL. Cellular protein was 100 µg and 160 to 220 µg in HSFs and SMCs, respectively. At the end of incubation, the cells were washed and extracted as in Fig 1. Cellular radioactivity (HSFs) in the presence of \( \beta \)-VLDL was 2.6±10^3 dpm/dish for up to 6 hours and 2.1±10^4 dpm after 24 hours of incubation. In the SMC experiment, these values were 3.1±10^3 at 6 hours and 2.4±10^3 dpm/dish at 24 hours of incubation. Values are mean±SEM of triplicate dishes.
TABLE 2. Effect of MP Medium on Cellular Uptake of β-VLDL Determined by [3H]CE Uptake or [3H]CE Formation From [3H]Oleic Acid in SMCs

<table>
<thead>
<tr>
<th>Medium</th>
<th>TC Radioactivity in cell, dpm</th>
<th>FC Radioactivity in cell, dpm</th>
<th>CE Radioactivity in cell, dpm</th>
<th>[3H]Cholesteryl oleate, nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>10324</td>
<td>6587±192</td>
<td>3737±301</td>
<td>0.83±0.03</td>
</tr>
<tr>
<td>DV</td>
<td>5050</td>
<td>3653±80</td>
<td>1397±225</td>
<td>0.48±0.05</td>
</tr>
<tr>
<td>MP</td>
<td>11008</td>
<td>5510±147</td>
<td>5498±450</td>
<td>1.74±0.02</td>
</tr>
<tr>
<td>DV</td>
<td>4801</td>
<td>2653±90</td>
<td>2148±26</td>
<td>0.97±0.03</td>
</tr>
</tbody>
</table>

MP indicates macrophage-conditioned; VLDL, very-low-density lipoprotein; CE, cholesteryl ester; SMCs, smooth muscle cells; TC, total cholesterol; FC, free cholesterol; DV, Dulbecco-Vogt. Nonlabeled cells were cultured as described in "Methods." The concentration of β-VLDL labeled with [3H]CE was 130 μg cholesterol/mL and 130 000 dpm/mL. After incubation with the labeled lipoproteins, the cells were washed sequentially with 0.2% albumin and phosphate-buffered saline and incubated with heparin 10 mg/mL at 4°C for 60 minutes. Cellular protein was 550 μg/dish. Experimental conditions for the [3H]CE-labeled part of the experiments are described in Fig 4, except that the β-VLDL concentration was 150 μg cholesterol/mL. The specific activity in the β-VLDL was 99% in CE; after incubation in either MP or DV medium for 24 hours without cells, no hydrolysis of the labeled CE was detected. Values are mean±SEM of triplicate dishes. All differences between DV and MP values in both experiments were statistically significant (P<0.01).

...sen because, as previously mentioned, at that time interval no effect of MP medium was yet evident.

We next compared the effect of MP and DV media by using two independent methods of evaluation of CE uptake and synthesis in the same experiment. Unlabeled SMCs were incubated with β-VLDL labeled with [3H]CE in the presence of MP or DV medium. In the two experiments shown in Table 2, the SMCs incubated with MP medium doubled their uptake of [3H]CE-labeled β-VLDL. The presence of 50% to 70% of cellular label in [3H]CIC indicated that MP medium did not interfere with cellular CE hydrolysis. In nonincubated β-VLDL, 99% of the CE radioactivity was 150 μg cholesterol/mL. The specific activity in the β-VLDL was 99% in CE; after incubation in either MP or DV medium for 24 hours without cells, no hydrolysis of the labeled CE was detected. Values are mean±SEM of triplicate dishes. All differences between DV and MP values in both experiments were statistically significant (P<0.01).

In our previous studies, we have shown that LPL derived from cultured heart cells or from bovine milk is very active in the transfer of CE or cholesteryl ester from different lipoproteins and liposomes to various cells.26-29 In view of the known presence of LPL in macrophage media, we wondered whether LPL might be responsible for the results obtained so far. LPL activity was inhibited in the MP medium either by addition of antiserum to LPL (99% inhibition) or by heating the medium to 56°C for 30 minutes (97% inhibition). The percent [3H]CE in SMCs exposed to MP medium was 16.6±0.2%; it was 17.8±0.1% when antiserum to LPL was added. Heating at 56°C for 30 minutes reduced the percent [3H]CE in SMCs exposed to MP medium to 9.0±0.3%. Similar results were obtained when the effect of MP medium in the presence of β-VLDL was tested on the formation of [3H]cholesteryl ester from [3H]cholesterol esterification from 2.6±0.1% in DV medium to only 9.0±0.3%. Similar results were obtained when the effect of MP medium in the presence of β-VLDL was tested on the formation of [3H]cholesteryl ester. Addition of antiserum to LPL to MP medium increased its effect on the formation of [3H]cholesteryl ester (Fig 4).

The next candidate considered for the MP effect was apoE, which is abundant in MP medium but either absent or extremely low in the medium of the macrophage-like cell line J774.30 Both MP and J774 media were found to have similar effects on [3H]cholesterol esterification (Fig 5). Addition of human recombinant apoE at various apoE/β-VLDL protein ratios to DV medium did not enhance [3H]cholesterol esterification (Fig 5).

The response of the FH-HSFs to β-VLDL in the presence of MP medium posed the question of whether the LDL receptor-related protein might be operative in this process. Lactoferrin is described as interfering with in vivo and in vitro binding of chylomicron remnants and...
β-VLDL by hepatocytes. Pretreatment of FH-HSFs with lactoferrin (2 mg/mL) for 1 hour before addition of β-VLDL and during the following 24-hour incubation period resulted in a reduction in the induced [3H]cholesterol esterification from 10.0±0.2% to 6.7±0.1%. No further reduction was seen with 4 mg/mL lactoferrin.

Next, the effect of MP medium on formation of [3H]CHOL from [3H]cholesterol in the presence of nonlabeled β-VLDL or LDL and the uptake and degradation of 123I-labeled lipoproteins were compared in the same experiment. The cells were not upregulated before the addition of the lipoproteins. In SMCs significant amounts of β-VLDL and LDL protein were metabolized during 24 hours of incubation (Table 3). In the presence of MP medium there were 157% and 37% increases in the metabolism of 123I-β-VLDL and 123I-LDL protein, respectively. In the cells obtained from two different LDL receptor-negative donors, 123I degradation products in the media were not detectable (ie, the same values were seen in the presence and absence of cells). The values given in Table 3 are of cell-associated 123I radioactivity only. In contradistinction to the negative results with the 123I-labeled lipoproteins, the effect of MP medium on the enhancement of [3H]cholesterol esterification in the presence of β-VLDL was seen in the cells of both FH donors (Table 3). Similar results were obtained in two additional comparisons.

Discussion

The present experiments were designed to study putative interaction between cultured aortic SMCs and macrophages that would affect cholesterol esterification in the SMC. We chose β-VLDL as a substrate because it is known to enhance CE accumulation in SMCs. SMCs were labeled with [3H]cholesterol, and formation of [3H]CE was determined after a 24-hour exposure to β-VLDL and either DV or MP medium. The finding of a more than twofold increase in the percent of labeled CE when cells exposed to MP medium in the presence of β-VLDL were compared with those in DV medium suggested the presence of a mediator(s) in the MP medium responsible for this effect. Since in the absence of added lipoproteins the percent of [3H]CE was not different in FH-HSFs or SMCs exposed to DV or MP medium, it was not plausible that the latter caused a direct activation of acyl coenzyme A:acyltransferase. The MP medium effect was also not caused by enhanced exchange of cellular [3H]CHOL with the added lipoprotein FC, as no significant differences were seen in the label

Table 3. Comparison of Effect of MP Medium on the Metabolism of 123I-Labeled Lipoproteins and on [3H]Cholesterol Esterification in the Presence of β-VLDL and LDL

<table>
<thead>
<tr>
<th>Medium</th>
<th>Lipoprotein</th>
<th>SMC</th>
<th>FH-HSF</th>
<th>LP Metabolized, μg/mg cell protein/24 h</th>
<th>Cholesterol ester, % of cellular label</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SMC</td>
<td>I</td>
<td>II</td>
<td>I</td>
</tr>
<tr>
<td>MP</td>
<td>β-VLDL</td>
<td>1.8±0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.06</td>
<td>13.4±0.1</td>
</tr>
<tr>
<td>DV</td>
<td>β-VLDL</td>
<td>0.7±0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.06</td>
<td>6.3±0.2</td>
</tr>
<tr>
<td>MP</td>
<td>LDL</td>
<td>2.6±0.09</td>
<td>&lt;0.04</td>
<td>&lt;0.03</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>DV</td>
<td>LDL</td>
<td>1.9±0.04</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>2.0±0.1</td>
</tr>
</tbody>
</table>

MP indicates macrophage-conditioned; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; FC, free cholesterol; SMCs, smooth muscle cells; FH-HSF, human skin fibroblasts from LDL receptor–negative donors with familial hypercholesterolemia; I and II, two different FH-HSF donors; DV, Dulbecco-Vogt. For experiments with 123I-labeled lipoproteins (123I-LP), nonlabeled cells were incubated with [3H]β-VLDL or LDL (25 μg protein/mL) for 24 hours. Thereafter, the medium was collected for determination of noniodide lipid metabolism products. The cells were washed with 0.2% albumin, phosphate-buffered saline, and heparin (see "Methods") and cell-associated protein radioactivity was determined after extraction with chloroform/methanol (1:1, vol/vol). 123I-LP metabolized in SMCs is the sum of 123I-LP catabolized and cell associated. For the experiments with [3H] cells were labeled with [3H]CHOL and exposed to nonlabeled lipoproteins (100 μg cholesterol/mL). Values are mean±SEM of triplicate dishes.
recovered in MP or DV medium in the presence of β-VLDL or LDL. The effect of the MP medium could have been due to a modification of the β-VLDL, as proposed in other studies in which β-VLDL conditioned with macrophages was shown to induce CE accretion in SMCs.37,38 However, in the present study a 24-hour preincubation of β-VLDL with the MP medium did not enhance cholesterol esterification after a subsequent 6 hours of incubation with SMCs. Another type of modification could have been peroxidation, but addition of butylated hydroxytoluene to the MP medium did not affect enhanced esterification in the presence of β-VLDL.

In view of the above, our first candidate for the MP effect was LPL, which has been shown to be secreted by macrophages39 and to enhance CE accretion in SMCs during chronic exposure to rabbit β-VLDL.40 It seems that LPL may influence interactions between lipoproteins and cells in various ways, depending on the parameters and lipoproteins studied. Thus, Aviram et al.41 suggest that LPL treatment of LDL results in a modification of the lipoprotein and thus enhances its uptake and degradation via the LDL receptor. Other investigators42 have studied the effect of LPL on the binding of chylomicrons to HepG2 cells and LDL receptor-negative fibroblasts and concluded that LPL strongly enhances the binding of apoE-containing lipoproteins to LDL receptor-related protein. However, when the effect of LPL on the binding of LDL or VLDL was investigated, it was found to be abolished by heparinase, an enzyme that specifically hydrolyzes heparan sulfate.43 These findings were corroborated and extended44 to show that LPL enhances not only the binding but also the uptake and degradation of 125I-LDL by HSFs. Enhanced degradation of LDL and especially of lipoprotein(a) occurred in the presence of LPL and could be reduced or abolished by heparitinase treatment for lipoprotein(a) or LDL, respectively.45 Our findings could not rule out participation of LPL in the MP medium effect on cholesterol esterification because the MP medium contained LPL activity. However, the lack of inhibition of the MP medium effect by antiserum to LPL does not lend much support to this possibility. It should be noted, however, that addition of relatively large amounts of LPL to DV as well as to MP medium did enhance cholesterol esterification in the SMCs.

Macrophages are known to synthesize and secrete apoE into the culture medium.46 It was shown that the addition of apoE to VLDL isolated from normalplasmic human plasma results in a marked increase in the uptake and degradation of the protein moiety of the VLDL by HSFs.47 However, when apoE was added to β-VLDL isolated from hypercholesterolemic rabbit plasma, no further stimulation of cholesteryl[14C]oleate formation was seen in HSFs.8 Our findings using β-VLDL agree with the latter report, as when apoE-enriched β-VLDL added to DV medium cholesterol esterification was not more enhanced than with β-VLDL alone. The present findings, that medium conditioned with J774 macrophages, which secrete little or no apoE,30 did enhance [3H]cholesterol esterification, lend additional support to the hypothesis that apoE may not be the "active" agent in MP medium. LDL receptor-negative cells (obtained from two different FH donors) responded to MP medium and β-VLDL with enhanced [3H]cholesterol esterification. Even though the response of the FH cells to MP medium (ie, the percent increase of cellular [3H]CE with MP medium compared with DV medium in the presence of β-VLDL) was the same as in normal HSFs, the maximal [3H]CE formation was much lower. When the FH fibroblasts were tested for uptake and degradation of 125I-labeled β-VLDL,125I protein degradation products in the media were nondetectable, and cell-associated protein radioactivity was low. However, as discussed by Kowal et al,8 the current assays for uptake and degradation of 125I-labeled β-VLDL do not allow reliable determinations of these parameters when their magnitude is low. It is of interest that after incubation with MP medium, the 125I-labeled cellular lipid derived from 125I-β-VLDL was increased twofold compared with cells incubated with DV medium. These results could suggest that a preferential uptake of lipid might have occurred in the presence of MP medium, analogous to our previous results with LPL and chylomicrons.48

The findings with the FH cells raised the question of the putative role of the LDL receptor–related protein in the MP effect on [3H]cholesterol esterification. In extrahepatic cells, the function of this receptor has been detected only in FH-HSFs lacking the LDL receptor.8,9 Lactoferrin, which possesses a cluster of four arginine residues, was shown to interfere with the in vivo uptake of chylomicron remnants and β-VLDL by the liver as well as in isolated liver cells.31,32,49 Since these lipoproteins bind to the LDL receptor–related protein,7 we used lactoferrin in the present study. When the cells were preincubated with up to 4 mg/mL lactoferrin, some reduction in the MP medium effect on cholesterol esterification with β-VLDL was found. However, the reduction was low in HSFs (5% to 18%), moderate in SMCs (33%), and highest in FH-HSFs (33% to 50%).

Another possible candidate to be considered for the MP medium effect could be a growth factor. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) have been shown to stimulate LDL receptor activity in aortic SMCs.50 Moreover, enhanced metabolism of LDL by PDGF was also shown in FH-HSFs.52 Human monocyte–derived macrophages were shown to secrete a factor into the culture medium that upregulated the LDL receptor in SMCs and HSFs but that did not enhance [3H]thymidine incorporation.11 Analogously, no growth stimulating activity was shown in conditioned medium from thioglycolate-elicited mouse peritoneal macrophages.53 It does not appear likely that growth factors play an important role in our system, as we did not observe significant differences in cell protein, and addition of bFGF to DV medium had no effect on cholesterol esterification.

Recently, a new VLDL receptor was described. The tissue expression of its mRNA was highest in heart, muscle, and adipose tissue, but it was barely detected in liver.54 The mature receptor protein was reported to have a striking homology to the LDL receptor, but in transfected cells it mediated uptake of VLDL and β-VLDL but not of LDL. It will be of interest to determine whether such a receptor might be responsible for the [3H]cholesterol esterification induced in FH-
HSFs by MP medium and β-VLDL. In conclusion, it seems that macrophage-conditioned medium may affect CE formation in SMCs and HSFs by LDL receptor-mediated and other pathways.

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References


6. Herz J, Hamans U, Rogge S, Myklebost O, Gauspehöf, Stanley KK. Surface location and high affinity for calcium of a 500-kd liver membrane protein closely related to the LDL- receptor suggest a physiological role as lipoprotein receptor. EMBO J. 1988;7:4119-4127.


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