Isoform-Dependent Cholesterol Efflux From Macrophages by Apolipoprotein E Is Modulated by Cell Surface Proteoglycans

Masumi Hara, Teruhiko Matsushima, Hiroaki Satoh, Naoyuki Iso-o, Hiroshi Noto, Masako Togo, Satoshi Kimura, Yoshiaki Hashimoto, Kazuhisa Tsukamoto

Objective—Apolipoprotein E (apoE) mediates cellular cholesterol efflux and plays a crucial role in the inhibition of atherogenesis. We investigated whether there is an isoform-specific difference in its function for cholesterol efflux from cholesterol-loaded RAW264.7 cells, a murine macrophage cell line that lacks endogenous apoE expression.

Methods and Results—When human apoE was expressed in RAW264.7 cells, apoE2 reduced cellular total cholesterol (TC) and esterified cholesterol (EC) levels significantly, whereas apoE3 and apoE4 had no effect. However, treatment of cells with 4-methylumbelliferyl-β-d-xyloside (β-DX) resulted in all 3 isoforms’ reducing cellular TC and EC contents significantly. We also investigated the effect of exogenously derived apoE on cholesterol efflux by utilizing the medium harvested from HeLa cells expressing apoE. ApoE2 and E3 reduced both cellular TC and EC contents significantly, whereas apoE4 did not. However, treatment of the cells with β-DX resulted in all 3 exogenously derived apoE isoforms’ reducing TC and EC contents significantly. The binding ability of apoE to heparan sulfate proteoglycans examined by heparinase I treatment revealed less binding ability of apoE2 compared with that of apoE3 or apoE4.

Conclusions—The present study clarified the differential cellular cholesterol–modulating effect of apoE isoforms in macrophages, which would be due to the difference in their binding to proteoglycans. (Arterioscler Thromb Vasc Biol. 2003;23:269-274.)

Key Words: apolipoprotein E • isoforms • cholesterol efflux • RAW264.7 cells • heparan-sulfate proteoglycans

Macrophages in the arterial wall play a crucial role in the progression of atherosclerosis.1 Macrophages are transformed to foam cells by their uptake cholesterol, form fatty streak lesions, and secrete cytokines that trigger the development of advanced complex atherosclerotic lesions.1 Cholesterol efflux is a pivotal mechanism that reduces the accumulation of cholesterol in macrophages and in which HDL has been proposed to play an important role.2 Apolipoprotein E (apoE) is a glycoprotein produced not only by the liver but also from macrophages.3,4 Besides its classic role in lipoprotein metabolism as a ligand for lipoprotein receptors,3,5 several studies have suggested additional roles for apoE in the protection against atherosclerosis. Overexpression of apoE in the liver of atherosclerosis-prone mice reduced atherosclerosis, accompanied by the deposition of hepatic-derived apoE in the atherosclerotic lesions.6,7 Macrophiage-specific expression of apoE in apoE-deficient mice resulted in reduced atherosclerosis,8 and bone marrow transplantation from apoE-deficient mice to normal mice increased atherosclerotic lesions9 without affecting plasma lipid profiles. Mice overexpressing apoE in the arterial wall show reduced atherosclerotic lesions.10 These studies suggest that both macrophage-derived and liver-derived apoE possess direct antiatherogenic properties.

There are 3 major isoforms in human apoE: apoE2, E3, and E4. Many studies have clarified differences among these isoforms in terms of their effects on lipoprotein metabolism and atherosclerosis.7,11–16 However, few studies have been performed to prove a differential effect of these isoforms on cholesterol-loaded macrophages. Cullen et al17 clarified a differential effect on cholesterol homeostasis in macrophages by apoE isoforms by using monocytes from humans homozygous for these isoforms. On the other hand, Smith et al18 did not find any difference in the efficiency for cholesterol efflux among these isoforms in RAW264.7 cells engineered to stably express the apoE isoform. Furthermore, no study has been performed to examine the isoform-specific cholesterol efflux by exogenously derived apoE under condition of nonendogenous apoE production in macrophages.

To address apoE isoform–specific effects on cholesterol metabolism in macrophages, we used adenovirus-mediated gene transfer to attain the same level of expression among the apoE isoforms. We also used 4-methylumbelliferyl 7-β-d-xyloside (β-DX)19,20 to inhibit the cellular production of...
cell-surface heparan sulfate proteoglycans (HSPGs) that would modulate the cholesterol-effluxing ability of apoE."#$%&'&"()'*+,-.0123456789:

Methods

Materials

RAW264.7 cells and HeLa cells were purchased from American Type Culture Collection (Manassas, VA) and the Health Science Research Resources Bank (Osaka, Japan), respectively. Adenoviral vectors AdE2, AdE3, and AdE4, encoding apoE2, E3, and E4 isoform cDNA, respectively, were used to express human apoE isoforms. AdLaCZ was used as a control adenovirus. Acetylated LDL was prepared from normal human LDL by following a method previously described.24

Determination of Multiplicity of Infection for Efficient Gene Delivery

RAW264.7 cells were infected with either 0, 80, or 240 multiplicity of infection (MOI) of AdLaCZ, and HeLa cells were infected with either 0, 1, or 100 MOI of AdLaCZ. Forty-eight hours after infection, cells were fixed with 0.5% glutaraldehyde and incubated overnight at 37°C with a solution containing 5 mmol/L K3 Fe(CN)6, 5 mmol/L K4 Fe(CN)6, 1 mmol/L MgCl2, and 1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal).

Expression of ApoE Isoforms in RAW264.7 Cells by Adenoviral Vectors

RAW264.7 cells were cultured with or without 1 mmol/L β-DX; infected with 240 MOI of AdE2, AdE3, AdE4, or AdLaCZ; and cholesterol-loaded with 100 µg/mL protein of acetylated LDL. Twenty-four hours later, cells were washed 3 times with PBS and cultured further for 48 hours in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.3% bovine serum albumin (BSA), and 0.3 mmol/L 8-Br-cAMP, with or without 1 mmol/L β-DX. For Western blot analysis, cells lysed in sample buffer and cell medium were stock at −80°C. For Northern blotting, 25 µg of cellular total RNA purified by the acid guanidinium-phenol-chloroform method was electrophoresed and blotted to nylon membranes, which were then hybridized with digoxigenin-labeled apoE3 cDNA probe followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody. The signals were detected with a chemiluminescence method, and the intensities of bands were quantified by using NIH Image software. After stripping the apoE probes, the membranes were reprobed with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

Studies With Endogenous ApoE Expression

RAW264.7 cells, cultured with or without 1 mmol/L β-DX for 48 hours, were either infected with 240 MOI of each adenoviral vector or left uninfected with an adenoviral vector and cholesterol-loaded with 100 µg/mL protein of acetylated LDL. For cholesterol-unloaded control cells, RAW264.7 cells were neither cholesterol-loaded nor infected with adenovirus. After another 24 hours, the cells were washed 3 times with PBS and cultured for an additional 36 hours in DMEM supplemented with 0.3% BSA and 0.3 mmol/L 8-Br-cAMP, with or without 1 mmol/L β-DX. After which, lipid extraction from cells was performed as described below. To evaluate the effect of 8-Br-cAMP on cholesterol efflux, some plates were cultured without 8-Br-cAMP and β-DX.

Studies With Exogenous ApoE

HeLa cells were either uninfected or infected with 100 MOI of each adenoviral vector. Twenty-four hours later, cells were washed 3 times with PBS and cultured for another 48 hours in DMEM supplemented with 0.3% BSA. The medium was then harvested, quickly centrifuged, and diluted with DMEM to assess the role of 8-Br-cAMP in cholesterol efflux. Thereafter, for the measurement of cellular lipid contents, cells were cultured for 36 hours. For the detection of cell surface–bound apoE, the cells were then cultured for 24 hours, washed 3 times with PBS, and incubated for another 4 hours with 0.5 mL DMEM with or without the addition of 2 U/mL heparinase I. The medium was quickly centrifuged, and the supernatant was preserved for Western blot analysis. The cells were harvested, and the cellular protein concentration was measured by the Lowry method.

Extraction and Quantification of Lipid From Cells

Cells were collected into tubes and washed 3 times with PBS. After a hexane/isopropanol mixture (3:2, vol/vol) was added, the tubes were vortexed and placed at room temperature for 30 minutes. After centrifugation, the supernatant was transferred to glass tubes and dried under nitrogen gas, followed by the measurement of cholesterol content; the pellet was used for measurement of cellular protein content. Total cholesterol (TC) and free cholesterol (FC) were measured by enzymatic methods, and protein concentration was measured by the Lowry method. After the adjustment of cellular TC and FC contents for cellular protein, the cellular esterified cholesterol (EC) content was calculated by subtracting the FC content value from the TC content value.

Western Blotting of ApoE

The medium collected from RAW264.7 cells, adjusted for the protein concentration of the cells, or aliquots of FPLC fractions were subjected to SDS–polyacrylamide gel electrophoresis. Proteins were blotted to nitrocellulose membranes and reacted with goat anti-human apoE polyclonal antibody, followed by incubation with peroxidase-labeled rabbit anti-goat IgG antibody. The protein bands were visualized with 4-chloro-1-naphthol as the substrate.

Results

Determination of MOI for Efficient Gene Delivery

The efficiency of gene delivery with adenoviral vectors was determined by X-gal staining of cells infected with AdLaCZ (Figure 1). In RAW264.7 cells, a few faintly stained cells were observed even at an MOI of 0, owing to the endogenous activity of galactosidase. As MOI increased, the proportion of stained cells increased and the intensity of the staining became stronger. At an MOI of 240, maximal gene expression (~70%) was observed. In HeLa cells, 100% of staining was observed at an MOI of 100. In consequence, for the subsequent experiments, an MOI of 240 and 100 was used for RAW264.7 cells and HeLa cells, respectively.

Expression of ApoE Isoforms in RAW264.7 Cells With Adenoviral Vectors

To examine whether infection at the same MOI of adenovirus resulted in the same level of expression, total RNA purified from RAW264.7 cells infected with either AdE2, E3, or E4 was subjected to Northern blot analysis (Figure 2A). The relative ratios of apoE mRNA to GAPDH mRNA were 1.12, 1.00, and 1.02 for apoE2, apoE3, and apoE4, respectively.
Western blot analysis of the medium revealed no difference in the protein levels among the apoE isoforms in the presence of β-DX (Figure 2B). However, without the addition of β-DX, little difference was observed in protein levels among the isoforms (Figure 2C); the relative apoE protein levels in the medium of apoE2 and apoE4 versus apoE3 were 1.93 and 1.31, respectively.

Effect of Endogenous ApoE Expression on Cellular Cholesterol
The cholesterol loading of RAW264.7 cells resulted in a 2- to 3-fold increase in cellular TC, FC, and EC contents (Figure 3). In the absence of β-DX, a significant reduction in the cellular TC and EC content was attained in cells expressing apoE2 (12.4% and 44.9% reduction for TC and EC, respectively, compared with LacZ) (Figure 3A). ApoE3 reduced cellular cholesterol content to a lesser degree than did apoE2, which was not significantly different from control. ApoE4 did not result in the reduction of cellular cholesterol. In the absence of 8-Br-cAMP, no change in cellular cholesterol was observed in cells expressing any apoE isoform (please see Figure 1A at http://atvb.ahajournals.org). Interestingly, in the presence of β-DX, all 3 apoE isoforms resulted in a significant reduction of cellular TC and EC contents (Figure 3B). Percentages for these reductions compared with LacZ control were as follows: 25.6% and 22.5% for TC and EC of apoE2, 33.2% and 43.1% for TC and EC of apoE3, and 28.7% and 40.6% for TC and EC of apoE4. In the presence of β-DX, the extent of reduction in cellular cholesterol was greatest with apoE3, followed by apoE4, and least in apoE2.

Effect of Exogenous ApoE on Cellular Cholesterol
FPLC analysis of the medium from HeLa cells infected with apoE adenovirus revealed that the apoE protein, regardless of
its isoform, distributed mostly on HDL and VHDL fractions and, to a lesser extent, on lipid-poor fractions (Figure 4).

In the absence of β-DX, apoE2- and E3-containing media significantly reduced cellular TC and EC contents (22.9% or 16.9% reduction for TC and 29.1% or 20.5% reduction for EC, respectively, compared with LacZ control) (Figure 5A). Medium containing apoE4 showed trends toward a reduction of TC and EC contents; however, no significant reduction was observed. When we compared the efficiency in the reduction of TC and EC among the 3 isoforms, apoE4 was less effective than apoE2 and E3, and apoE2 showed a tendency in the increased reduction for TC and EC compared with apoE3. The reduction in cellular cholesterol by apoE isoforms was not observed in the absence of 8-Br-cAMP (please see Figure IB at http://atvb.ahajournals.org).

In the presence of β-DX, all 3 exogenous apoE isoforms resulted in a significant reduction of cellular TC and EC contents (Figure 5B), which was the case with endogenous apoE isoforms. However, the differential effect among the isoforms was not found in this experiment with exogenous apoE.

**Detection of Cell Surface–Bound ApoE by Using Heparinase**

To elucidate whether the binding ability to cell surface HSPGs differs among apoE isoforms, RAW264.7 cells were incubated with the medium harvested from HeLa cells for 24 hours, which were then washed and incubated with 2 U/mL heparinase I. The catalyzing activity of heparinase I for HSPGs was confirmed by additional experiments with lactoferrin (data not shown). In the absence of β-DX, the amount of apoE released by heparinase I increased in the following order: E2 < E3 < E4 (Figure 6A and B). On the other hand, when the production of cell surface HSPGs was inhibited by β-DX, release of apoE protein from the cell surface by heparinase I was not observed (Figure 6C).

**Discussion**

It has been difficult to induce macrophage cell lines to express any genes by the DNA transfection method. Adenoviral vectors are good tools for gene transfer into any cells that express specific integrins and the coxsackievirus and adenovirus receptor. Another benefit of using adenoviral vectors for gene transfer is that it is possible to make the level of expression similar among different genes by using the same promoter and infecting viruses at the same MOI. As was shown in Results, good efficiency of infection up to 70% was obtained by adenovirus-mediated gene transfer to RAW264.7 cells, and infection at the same MOI resulted in the same levels of expression among the 3 apoE isoforms. The levels of apoE protein expression analyzed with the cellular proteins were almost the same among the 3 isoforms, confirming the same levels of expression in the cells (data not shown).
regardless of whether it was derived from outside or inside the cells. ApoE3 was less effective than apoE2 when synthesized in the cells; however, when exogenously derived, it reduced cellular cholesterol content significantly, which still showed a reduced tendency in cholesterol efflux compared with apoE2. ApoE4 had no significant effect on the cellular cholesterol content. The binding of apoE to cell surface HSPGs increased in the order E2 < E3 < E4. These observations, together with the findings obtained from the experiments without HSPGs, suggest that the secreted and exogenously derived apoE4 protein, as well as a portion of apoE3 protein, would be captured by the cell surface HSPGs and taken up and/or degraded by the cells. Support for this concept is the report by Lucas et al., which demonstrated that the apoE sequestered in the pericellular proteoglycan matrix is susceptible to cellular degradation. Ji et al. reported that the binding ability of apoE-VLDL to HSPGs depends on the apoE isoforms in the order E4 > E3 > E2. This finding is consistent with ours, considering that the lipoproteins examined in their study are apoE-VLDL particles and those in ours are HDLs and lipid-poor apoEs. The size and composition of lipoprotein particles would modulate the presentation of apoE to HSPGs, thereby leading to the different binding ability to HSPGs among the different lipoprotein classes.

The LDL receptor is another candidate that mediates the binding of apoE to the surface of macrophages as well as HSPGs. The binding activity of apoE2 to the LDL receptor is < 2% when compared with apoE3 and E4; however, our data without HSPGs with exogenous apoE revealed that there is no difference in the efficiency of cholesterol efflux among the 3 apoE isoforms. This finding suggests that the LDL receptor is not a key player in the cholesterol efflux mediated by the cell surface–bound apoE or apoE-containing HDLs in macrophages.

The present study demonstrated that the ability of apoE to promote cholesterol efflux from macrophages is not fundamentally different among the apoE isoforms. However, in the physiological setting wherein HSPGs are produced, apoE2 is most and apoE4 least effective. Epidemiological studies have clarified that the incidence of coronary heart disease is generally lower in humans carrying at least 1 apoE2 allele than those possessing other apoE genotypes, and the allele for apoE4 is an independent risk factor for coronary heart disease. We speculate that under normolipidemic conditions, in which the insult by atherogenic lipoproteins is low, apoE2 functions most effectively for the reduction in cellular cholesterol among the 3 isoforms, thus protecting humans who possess apoE2 from atherosclerosis; on the other hand, apoE4 is least effective in the efflux and would lead to the increased atherosclerosis in humans who carry the apoE4 allele.

ApoE has been proposed to possess several biologic properties that could contribute to antiatherogenic effects. The apoE polymorphism has been shown to affect some of these functions. In addition, apoE isoforms have been proved to possess differential direct cellular functions. More investigations into the differential biologic effects of the isoforms would improve our understanding of the clinical observations that identified the differential effects of apoE isoforms on atherogenesis.
In summary, we demonstrated that the ability of apoE isoforms to promote cholesterol efflux from macrophages is fundamentally similar among the apoE isoforms, regardless of whether it is derived from outside the cell or synthesized inside the cell. Nevertheless, in the physiological setting where proteoglycans are produced by macrophages, the cell surface HSPGs would modulate the ability of apoE isoforms to promote cholesterol efflux, probably through the differential binding ability of each apoE isoform to HSPGs.

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