Independent Regulation of Cholesterol Incorporation into Free Apolipoprotein-mediated Cellular Lipid Efflux in Rat Vascular Smooth Muscle Cells*

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Cholesterol was poorly available to free apolipoprotein (apo)A-I-mediated cellular lipid efflux from cholesterol-loaded rat vascular smooth muscle cells generating cholesterol-poorer pre-β-HDL particles than those generated from macrophages by the same reaction (Li, Q., Komaba, A., and Yokoyama, S. (1993) Biochemistry 32, 4597–4603). The factors known to induce transformation of the smooth muscle cells into a macrophage-like stage were used in order to modulate this reaction, such as human platelet-derived growth factor, macrophage colony-stimulating factor, and phorbol 12-myristate-13-acetate (PMA). When the cells were stimulated by PMA following the pretreatment with platelet-derived growth factor plus macrophage colony-stimulating factor, cholesterol efflux mediated by free apoA-I increased 3-fold without changing phospholipid efflux, resulting in generation of pre-β-HDL particles more rich in cholesterol. This treatment had only a little or no effect on apparent cellular cholesterol efflux to HDL or lipid microemulsion, respectively. Overall cellular free cholesterol pool size was unaffected by the treatment, and probing by extracellular cholesterol oxidase did not detect gross change in the cellular surface cholesterol. This specific enrichment of cholesterol in the apoA-I-mediated cellular lipid efflux was reversed by protein kinase C inhibitors. Measurement of intracellular cholesterol esterification suggested that PMA induced translocation of intracellular cholesterol to a specific pool for apoA-I-mediated efflux, and a protein kinase C inhibitor reversed this effect.

When cholesterol is transported from the peripheral cells to the liver for its biological degradation, the first step of the pathway is efflux of cellular cholesterol to plasma lipoproteins. High density lipoprotein (HDL)1 is believed to play a primary role in this step by “accepting” the cellular cholesterol. Cellular free cholesterol is exchangeable with extracellular lipoprotein surface by a nonspecific physicochemical mechanism such as its diffusion through aqueous phase or direct collision between the membranes (Rothblat and Phillips, 1982; Johnson et al., 1986; Karlin et al., 1987; Johnson et al., 1988). The net efflux could therefore be induced by a gradient of cholesterol content between cell membrane and lipoprotein surface, so that cholesterol esterification on HDL may play an important role in such a mechanism (Murphy, 1962; Glomset, 1968; Ray et al., 1980; Fielding and Fielding, 1981; Czarnecka and Yokoyama, 1995). Specific function of HDL to accommodate cholesterol may also be attributed to physicochemical properties of its surface (Stein et al., 1986; Phillips et al., 1987). On the other hand, a HDL-binding protein(s) on the cell surface is proposed to play a specific role being linked to intracellular signal transduction that results in mobilization of intracellular cholesterol to cellular surface (Graham and Oram, 1987; Slotte et al., 1987; Mendez et al., 1991; McKnight et al., 1992).

Recent development of the investigation in this field shed light for further understanding of the mechanism for cellular cholesterol efflux. Fielding and co-workers discovered that cellular cholesterol appeared in a specific HDL fraction, pre-β-HDL, in the very early stage of its efflux and then was transferred to other lipoproteins (Castro and Fielding, 1988; Miida et al., 1990, 1992) and proposed that this lipoprotein fraction is the most efficient “acceptor” of cellular cholesterol. Later, we demonstrated that lipid-free apolipoproteins having multisequences of amphiphilic helaxx mediate the net efflux of cholesterol and phospholipid from various types of cells, generating new HDL-like particles with these cellular lipids (Hara and Yokoyama, 1991, 1992; Hara et al., 1992; Komaba et al., 1992). The particles were consistent with pre-β-HDL with respect to the chemical and physical properties. Other groups have also reported similar data using lipid-free apolipoproteins (Bielicki et al., 1992; Forte et al., 1993). These results may explain why cellular cholesterol appears in pre-β-HDL fraction in the very early stage of the efflux (Castro and Fielding, 1988; Miida et al., 1990, 1992), pre-β-HDL particles are newly generated with cellular lipid by apolipoproteins dissociated from HDL, rather than the particles in the medium preferably “accepting” cholesterol from cellular surface.

The reaction can be carried out by a number of amphiphilic helical apolipoproteins (Hara and Yokoyama, 1991; Hara et al., 1992; Bielicki et al., 1992) or their model peptides (Mendez et al., 1994), and it may induce specific translocation of intracellular cholesterol that would render cholesterol readily available for the efflux (Mendez et al., 1994). The involvement of a protein binding site(s) for such apolipoproteins has not clearly been identified on cellular surface. However, many circumstantial evidences may support a potential role of such a site(s) in

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§ The abbreviations used are: HDL, high density lipoprotein(s); apo, apolipoprotein; PBS, phosphate-buffered saline; LDL, low density lipoprotein; PDGF, platelet-derived growth factor; MCSF, macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine.
this reaction. Apparent $K_m$ values for apolipoprotein-mediated lipid efflux were as low (Hara and Yokoyama, 1991; Komaba et al., 1992) as that of the LDL-receptor interaction ($~10^{-8}$ M) (Brown and Goldstein, 1986), and lower than that of the lipid-apolipoprotein interaction ($~10^{-7}$ M) (Tajima et al., 1983; Yokoyama et al., 1985), indicating some type of specific interaction. Proteolytic treatment of cellular surface resulted in complete inhibition of this pathway (Li et al., 1995). Furthermore, specific lack of interaction with lipid-free apoA-I in fibroblasts from the patients with Tangier disease strongly suggested that there is a genetically defined cellular interaction site for lipid-free apolipoproteins, and that this reaction is a major source of plasma HDL (Francis et al., 1995).

Thus, free apolipoprotein-mediated cellular cholesterol efflux seems to be a distinct pathway from nonspecific physicochemical cholesterol exchange reaction between cellular surface and lipoproteins. This may lead us to the hypothesis that HDL-mediated lipid efflux includes two distinct mechanisms. One is physicochemical lipid exchange between the cell and HDL surface in which gradient of cholesterol between the two surfaces causes the net efflux and therefore cholesterol esterification on HDL may act as its driving force. The other is generation of new pre-$\beta$-HDL with cellular lipids, as apolipoproteins locally dissociate from HDL surface to interact with the cellular surface (Hara and Yokoyama, 1991, 1992). The maximum contribution of the free apolipoprotein-mediated portion could be as much as 40% of apparent HDL-mediated efflux (Komaba et al., 1992; Francis et al., 1995).

Interestingly, vascular smooth muscle cells are very resistant to the free apolipoprotein-mediated cholesterol efflux (Komaba et al., 1992) due to generation of cholesterol-poor pre-$\beta$-HDL (Li et al., 1993). This was specific to lipid-free apolipoprotein-mediated cholesterol efflux, whereas little significant difference was found among cell lines in the nonspecific cholesterol efflux mediated by low density lipoprotein (LDL) or lipid microemulsions (Li et al., 1993). The low cholesterol/phospholipid ratio in the HDL-mediated lipid efflux from smooth muscle cells implies that free apoA-I-mediated mechanism is in fact involved in cellular lipid efflux to HDL (Li et al., 1993). These findings indicated that cell-specific intracellular factors are involved in regulation of cholesterol incorporation into the cellular lipid efflux mediated by apolipoprotein-cell interaction.

Vascular smooth muscle cells are known to be stimulated by certain growth factors and transformed to different stages, and such transformation may be related to its roles in development of vascular atherosclerotic lesions. The smooth muscle cells isolated from arteriosclerotic lesion were found to express the c-fms gene encoding the receptor for macrophage colony-stimulating factor (MCSF) (Inaba et al., 1992a). In vitro, platelet-derived growth factor (PDGF) BB-chain homodimer stimulates the smooth muscle cells to induce c-fms gene as well as the scavenger receptor gene, as transforming the cells to a macrophage-like stage (Inaba et al., 1992b). On the other hand, in the undifferentiated macrophage cell line cell THP-1, phorbol esters are known to induce differentiation of the cells with respect to many macrophage activities, including expression of the scavenger receptor (Hara et al., 1987; Takata et al., 1989) and apolipoprotein E and lipoprotein lipase (Tajima et al., 1985; Menju et al., 1989). Hence, the in vitro transformation of the cells by these factors may modulate the cell specific factors involved in apolipoprotein-mediated lipid efflux and would provide an important model to study the intracellular mechanism for cholesterol efflux by this particular pathway.

Thus, we undertook the study of lipid-free apoA-I-mediated lipid efflux from rat vascular smooth muscle cells in comparison to the apparent cholesterol efflux to lipoproteins and lipid microemulsions under the influence of stimulation of the cells by the growth factors and phorbol esters.

**EXPERIMENTAL PROCEDURES**

**Lipoprotein, Apolipoprotein, and Lipid Microemulsion—**HDL was isolated from fresh human plasma as the fraction with a density of 1.063–1.21 g/ml in NaBr. The purity of the lipoprotein preparation was verified by electrophoresis in 5% agarose and by apolipoprotein composition analysis in polyacrylamide gel electrophoresis in the presence of 0.5% sodium dodecyl sulfate, in order to assure no contamination of LDL or apoB. The particular preparation used was a mixture of HDL$_2$, and HDL$_3$, in approximately 1 to 1 protein ratio, determined by the extracted lipid and cellular cholesteryl ester uptake was calculated. Data represent mean ± S.E. for triplicated assay. Cellular protein was 239.6 ± 28.2 μg/dish.

| Treatment of the cell | Cholesteryl ester uptake (μg cholesterol/mg cell protein) |
|-----------------------|----------------------------------------------------------|
| Control               | 2.00 ± 0.07                                              |
| MCSF                  | 2.26 ± 0.15                                              |
| PDGF                  | 3.28 ± 0.10                                              |
| PDGF + MCSF           | 3.98 ± 0.21                                              |

**Uptake of cholesteryl ester of acetylated LDL by rat vascular smooth muscle cells stimulated by growth factors**

The cells were treated with PDGF, MCSF, or both for 24 h as described in the text. In the presence of acetylated LDL containing radio-labeled cholesteryl ester (54296 dpm/μg cholesterol moieties), 26 μg as protein, the radioactivity in the cells was counted for the extracted lipid and cellular cholesteryl ester uptake was calculated. Data represent mean ± S.E. for triplicated assay. Cellular protein was 239.6 ± 28.2 μg/dish.
linysulfonil)-2-methylpyperazine (H-7) (all obtained from Sigma) were also added to this 45-min period as protein kinase C inhibitors, in the attempt to reverse the effect of PMA (Hannun et al., 1986; Tamaoki et al., 1986; Hida et al., 1984). Sphingosine was suspended with 2 mM bovine serum albumin solution in PBS to give its concentration of 25 mM, and added to the medium to make final concentration of 5–40 μM at the step of the PMA treatment (Hannun et al., 1986). Staurosporine was dissolved in dimethyl sulfoxide as 4 mM and added to the medium to make final concentration of 0.5–50 nM at the stage of the PMA treatment (Tamaoki et al., 1986). H-7 was dissolved in water as 5 mM and added to the medium to make final concentration of 12.5–100 μM (Hida et al., 1994).

Efflux of Cellular Cholesterol and Choline Phospholipid—After the pretreatment, the cells were washed with the medium for three times and then incubated with HDL, apoA-I, and lipid microemulsion in the presence of 2 mg/ml bovine serum albumin. Radioactivity of free and esterified cholesterol, phosphatidylcholine and sphingomyelin in the medium, and cells was then counted after the lipid was extracted and separated by thin layer chromatography (Hara and Yokoyama, 1991, 1992). Radioactivity of each lipid in the medium in the presence of bovine serum albumin alone was less than 5% of the level induced by HDL as 50 μg of protein, and this was subtracted from each data point as a background (Komaba et al., 1992; Li et al., 1993). Specific radioactivity of cellular total cholesterol and each phospholipid was calculated based on their chemical amount, measured by an enzymatic fluorometric assay, respectively, and used for calculation of the amount of the efflux into the medium (Hara and Yokoyama, 1991; Komaba et al., 1992; Li et al., 1993). The validity of this method was verified by quantification of cholesterol with gas-liquid chromatography (Hara and Yokoyama, 1991). In some experiments, the efflux was expressed as radioactivity of each lipid in the medium standardized for cellular pool of the respective cellular lipid in each culture dish specimen (Komaba et al., 1992) or radioactivity per cellular protein. Cellular protein of each dish was measured by the method of Lowry et al. (1951). The experimental data points were duplicated unless otherwise specified, mostly within a 10% error margin, and the values in the figures represent the averages unless otherwise specified.

Other Methods—The medium was analyzed for the lipid efflux product by using density gradient ultracentrifugation in sucrose as described earlier (Hara and Yokoyama, 1991). Sucrose solution of density 1.30 (0.7 ml) was overlaid with the solution of density 1.10 (1.2 ml), and then the culture medium (1.2 ml) was overlaid on the top in a 3-ml quick-seal centrifuge tube for a Beckman TL100.3 rotor. After centrifugation at 99,000 rpm in a TL100 ultracentrifuge for 16 h at 4 °C, 200-μl fractions were collected from the bottom. For each fraction, density was measured and radioactivity of free cholesterol and phosphatidylcholine was counted after lipid extraction and thin layer chromatography. The weight of each lipid was calculated by using the specific radioactivity of the respective lipid in a cellular pool (Hara and Yokoyama, 1991; Li et al., 1993).

Cellular cholesterol was oxidized by extracellular cholesterol oxidase (Sigma) in order to assess accessibility from the cellular surface, and the oxidized product, cholestene, was analyzed by thin layer chromatography after extracting the cellular lipid, as described in detail previously (Lange and Ramos, 1983; Li et al., 1993).

Intracellular cholesterol esterification activity was measured for the cholesterol-loaded cells after treatment with growth factors and PMA, according to the method previously described (Francis et al., 1993). The cells were treated in the same manner as the efflux experiment for cholesterol loading with cationized LDL, the growth factor treatment, and PMA stimulation in the presence and absence of H-7. After washing the medium containing [1-14C]oleic acid (57 mCi/mmol, Amersham), 1.5 μCi (26 nmol) was added to each culture dish. After a 1-h incubation at 37 °C, the cells were washed with ice-chilled medium and lipid was extracted for the analysis by thin layer chromatography. The radioactivity in cholesteryl ester fraction was counted.

**RESULTS**

Lipid efflux profile was compared between rat aortic smooth muscle cells and mouse peritoneal macrophages. The efflux to lipid microemulsion represents nonspecific physicochemical pathways and apoA-I-mediated efflux represents the efflux pathway by generating new pre-β-HDL particles with cellular lipid. The condition was chosen for the V_max of the efflux rate at the 24-h incubation (Hara and Yokoyama, 1991, 1992), and the results shown in Table II were consistent with our previous observation (Li et al., 1993). In nonspecific pathway (to lipid microemulsion), the rate of cholesterol efflux was much higher than that of phospholipid and the ratio of cholesterol to total phospholipid was very similar for the two types of cell, giving a weight ratio around 4. In contrast, phospholipid efflux was substantially high in the apoA-I-mediated efflux from both cells. Specific difference was observed in cholesterol efflux between smooth muscle cells and macrophages. Cholesterol was very poorly incorporated from smooth muscle cells into this pathway, making its weight ratio to phospholipid substantially lower than the efflux from macrophages, 0.24 versus 1.57. In lipid efflux to HDL, the value of this parameter was intermediate between the emulsion-mediated and apoA-I-mediated pathways.

Smooth muscle cells were exposed to the potential stimuli to induce macrophage-like functions. Such factors as PDGF, MCSF, and PMA were used in an attempt to modulate lipid efflux profile for the reasons mentioned earlier, and the results are shown in Table III. When each of the three factors was individually used, there was no effect on the cholesterol efflux from the cells either by HDL or apoA-I. Since PDGF is known to induce MCSF receptor (Inaba et al., 1992b), the two growth factors were used together, but no additional effect was found by this combination. When the cells were stimulated by PMA following the treatment with growth factors, cholesterol efflux was somewhat enhanced from the PDGF-pretreated cells but not from the MCSF-treated cells. No increase was observed in the apparent cholesterol efflux to HDL in this condition. Finally, when both growth factors were used together in the pretreatment for the PMA-stimulation, the efflux of cholesterol mediated by lipid-free apoA-I was enhanced 3-fold. The increase was only 30% with the efflux to HDL by the same treatment. In contrast to the results for the cholesterol efflux, phospholipid efflux mediated by apoA-I was not significantly affected by the treatment with PDGF/MCSF and PMA. Total cellular protein has not reached significant difference by any of these treatments (Table III). To examine potential contribution to the efflux of intracellular cholesteryl ester hydrolysis as the

| Emulsion       | Smooth muscle cells | Macrophages |
|----------------|---------------------|-------------|
| Cholesterol    | 187 ± 33            | 275 ± 16    |
| Phosphatidylcholine | 27.4 ± 5.6       | 58.9 ± 4.2  |
| Sphingomyelin  | 15.7 ± 4.2          | 9.7 ± 1.2   |
| Cholesterol/phospholipid ratio | 4.34 | 4.01 |

| ApoA-I          | Smooth muscle cells | Macrophages |
|-----------------|---------------------|-------------|
| Cholesterol    | 511 ± 69            | 239 ± 25    |
| Phosphatidylcholine | 175 ± 16       | 126 ± 20   |
| Sphingomyelin  | 31.3 ± 4.2          | 26.0 ± 4.3  |
| Cholesterol/phospholipid ratio | 0.24 | 1.57 |

| HDL             | Smooth muscle cells | Macrophages |
|-----------------|---------------------|-------------|
| Cholesterol    | 341 ± 31            | 438 ± 11.5  |
| Phosphatidylcholine | 135 ± 8            | 128 ± 16.1 |
| Sphingomyelin  | 49 ± 2.8            | 36 ± 9.7    |
| Cholesterol/phospholipid ratio | 1.85 | 2.67 |
been poor in smooth muscle cells (Li et al., 1993). The efflux of lipids was observed in the presence of apoA-I (10 μg) or HDL (100 μg of protein) for 24 h. The lipid efflux value is percentage of cellular pool of respective lipid, and cellular protein is μg/dish, representing mean ± S.E. of four data points. FC/TC, free/total cholesterol ratio (%) in the cell in radioactivity before the efflux mediators were added. Statistical significance for the difference from the control experiments: *, p < 0.01; **, p < 0.001.

| Cellular protein (μg/dish) | Control | PDGF | MCSF | PDGF/MCSF |
|---------------------------|---------|------|------|-----------|
| PMA (+)                   | 306 ± 32| 322 ± 34| 292 ± 26| 338 ± 46 |
| PMA (+)                   | 290 ± 22| 318 ± 50| 260 ± 50| 364 ± 78 |
| FC/TC (%) in PMA (+)      | 48 ± 4 | 49 ± 4 | 46 ± 5 | 45 ± 4 |

| Cholesterol efflux (% of cellular FC pool) | Control | PDGF | MCSF | PDGF/MCSF |
|-------------------------------------------|---------|------|------|-----------|
| PMA (+)                                   | ApoA-I  | 5.5 ± 1.8 | 5.3 ± 0.7 | 4.7 ± 1.4 | 5.0 ± 0.7 |
|                                          | HDL     | 18.2 ± 2.1 | 19.0 ± 2.4 | 16.2 ± 2.8 | 19.1 ± 2.5 |
|                                          | ApoA-I  | 4.9 ± 1.3 | 8.0 ± 2.5* | 5.1 ± 0.5 | 14.4 ± 1.3** |
|                                          | HDL     | 21.3 ± 3.2 | 21.8 ± 3.4 | 17.6 ± 3.0 | 29.5 ± 2.9* |

| Phosphatidylcholine efflux (% of cellular pool) | Control | PDGF | MCSF | PDGF/MCSF |
|------------------------------------------------|---------|------|------|-----------|
| PMA (+)                                        | ApoA-I  | 20.6 ± 5.3 | 29.3 ± 6.1 | 21.6 ± 1.3 | 27.8 ± 2.8 |
|                                          | HDL     | 8.2 ± 3.1 | 8.7 ± 1.9 | 7.7 ± 2.5 | 8.6 ± 2.1 |
|                                          | ApoA-I  | 19.2 ± 4.4 | 28.2 ± 8.0 | 20.2 ± 4.5 | 25.3 ± 7.6 |
|                                          | HDL     | 10.2 ± 2.0 |               |               | 11.4 ± 2.2 |

Table IV shows the comparison of the effect on the apoA-I-mediated cholesterol efflux pathway and a nonspecific physicochemical efflux pathway to lipid microemulsion of the treatment of the smooth muscle cells with the growth factors and PMA. While the enhancement was demonstrated for the apoA-I-mediated efflux, there was no influence of the combined treatment.

![Fig. 1](image)

**FIG. 1.** Time course of lipid efflux from smooth muscle cells induced by apoA-I (top) and by HDL (bottom). Circles, control; triangles, treatment with PDGF and MCSF plus PMA. Left panels, cholesterol efflux. Right panels, phosphatidylcholine efflux. The cells were labeled for cholesterol and choline phospholipid as described in the text. The lipid efflux was monitored up to 24 h in the presence of 10 μg of apoA-I or 100 μg of HDL as protein in the 1-ml medium. Cellular protein per dish was 310.5 ± 19.4 μg. Radioactivity of cellular free cholesterol per dish was 20842 ± 2840 dpm, and cellular free cholesterol was 48.5 ± 2.2% of total cellular cholesterol in radioactivity. Radioactivity of cellular phosphatidylcholine was 6516 ± 668 cpm/dish.

In order to observe whether this specific increase of cholesterol incorporation into apoA-I-mediated cellular lipid efflux pathway is related to gross change in cellular cholesterol distribution, surface cholesterol of smooth muscle cells was probed by extracellular cholesterol oxidase. Fig. 4 illustrates the percentage of oxidized cellular cholesterol in the control and activated conditions. There was no difference in cellular surface cholesterol observed by this procedure either in the initial rate of the oxidation or the end point of the reaction between the control cells and the cells after the combined treatment.
Regulation of Apolipoprotein-mediated Cholesterol Efflux

Free cholesterol was 42.3% in the cells, while the effect on phosphatidylcholine efflux by PMA from the growth factors-treated cells was almost completely reversed, while the effect on phosphatidylcholine efflux by PMA, respectively. In contrast, the effect of PMA and H-7 on intracellular cholesterol efflux was already high, and no significant effect of any of these treatments was observed on cholesterol and phospholipid efflux either mediated by HDL or free apoA-I (data not shown). Further incubation with phorbol ester (PMA) of the pretreated cells with the growth factors, which led to a specific effect on smooth muscle cells as described above, did not show any further differential effect on the lipid efflux, either among the different pretreatment groups with the growth factors or between the efflux mediated by apoA-I and HDL (Table V).

Finally, the experiment was performed for the treatment of mouse peritoneal macrophages with PDGF and MCSF, individually or in combination. Cholesterol efflux from the control cells was already high, and no significant effect of any of these treatments was observed on cholesterol and phospholipid efflux either mediated by HDL or free apoA-I (data not shown). Further incubation with phorbol ester (PMA) of the pretreated cells with the growth factors, which led to a specific effect on smooth muscle cells as described above, did not show any further differential effect on the lipid efflux, either among the different pretreatment groups with the growth factors or between the efflux mediated by apoA-I and HDL (Table V).

**FIG. 2.** Dose dependence of the apoA-I-mediated lipid efflux from smooth muscle cells. Circles, control; triangles, treatment with PDGF and MCSF, plus PMA. Panel A, cholesterol efflux; panel B, phosphatidylcholine efflux. The cells were labeled for cholesterol and choline phospholipid as described in the text, treated with growth factors and PMA, and the lipid efflux was observed in the presence of various amounts of apoA-I for 24 h. Cellular protein per dish was 346.8 ± 15.3 μg. Radioactivity of cellular free cholesterol per blank dish was 22526 ± 2697 cpm, and that of cellular phosphatidylcholine was 19539 ± 2021 cpm. Cellular free cholesterol was 42.3 ± 2.6% of total cellular cholesterol in radioactivity.

**FIG. 3.** Density gradient analysis of the product in the culture medium after incubation of apoA-I with the smooth muscle cells, control (left panel) and activated (right panel). Smooth muscle cells were activated by PDGF, MCSF, and PMA as described in the text. ApoA-I, 30 μg/ml, induced cellular lipid efflux for 24 h. The medium was then analyzed in sucrose density gradient ultracentrifugation as described in the text. Specific radioactivity of cholesterol and phosphatidylcholine was obtained for respective cellular lipid pool and used for calculation of weight amount of cholesterol and phosphatidylcholine in each density fraction of the medium. Cellular protein was 375.2 ± 31.9 μg/dish, and total cellular cholesterol was 54.2 ± 0.55 μg. Free cholesterol was 72.3 ± 0.5% of total cellular cholesterol. The data are expressed as weight percentage of each lipid in each fraction to total phosphatidylcholine in the medium. Circles, cholesterol; triangles, phosphatidylcholine; solid line without symbol, density.

**Fig. 5 further demonstrates the effect of protein kinase C inhibitors on the enhancement of apoA-I-mediated cellular lipid efflux from smooth muscle cells. Both sphingosine and staurosporine suppressed the cholesterol efflux in a dose-dependent manner (left panel). The maximum inhibition by sphingosine and staurosporine was 81% and 47% of the increment of cholesterol efflux by PMA, respectively. In contrast, staurosporine showed no significant effect on phosphatidylcholine efflux mediated by apoA-I (right panel). A specific inhibitor of cyclic nucleotide-dependent protein kinase C, H-7, was used in a similar manner (Fig. 6). The increase of apoA-I-mediated cholesterol efflux by PMA from the growth factors-treated cells was almost completely reversed, while the effect on phospholipid efflux was negligible (left panel). The same compound demonstrated no effect on nonspecific lipid efflux from the similarly activated smooth muscle cells to lipido microemulsions (right panel).

Intracellular cholesterol esterification activity was determined in the same stages of activation of cholesterol-loaded smooth muscle cells by the growth factors and PMA as used for lipid efflux experiments (Fig. 7). Incorporation of radiolabeled oleate into cholesteryl ester was not significantly changed by the cellular treatment with both growth factors. Adding PMA for short periods (45 min) significantly decreased the reaction, suggesting the decrease of cholesterol pool available for this reaction. The presence of H-7 completely reversed the effect of PMA. Thus, the effect of PMA and H-7 on intracellular cholesterol esterification was reciprocal to the effect of these compounds on apoA-I-mediated cholesterol efflux from the growth factor-treated vascular smooth muscle cells.

Finally, the experiment was performed for the treatment of mouse peritoneal macrophages with PDGF and MCSF, individually or in combination. Cholesterol efflux from the control cells was already high, and no significant effect of any of these treatments was observed on cholesterol and phospholipid efflux either mediated by HDL or free apoA-I (data not shown). Further incubation with phorbol ester (PMA) of the pretreated cells with the growth factors, which led to a specific effect on smooth muscle cells as described above, did not show any further differential effect on the lipid efflux, either among the different pretreatment groups with the growth factors or between the efflux mediated by apoA-I and HDL (Table V).

**DISCUSSION**

After short periods of incubation, PMA stimulated the cholesterol efflux by free apoA-I from the rat vascular smooth muscle cells pretreated with growth factors. The effect was

**FIG. 4.** Oxidation of cholesterol in smooth muscle cells by extracellular cholesterol oxidase. Circles, control; triangles, treatment with PDGF and MCSF, plus PMA. Rat vascular smooth muscle cells were loaded with radiolabeled cholesterol as described in the text. After pretreatment with the growth factors and PMA, cells were incubated with cholesterol oxidase (3 IU/ml) for various periods of time and the radioactivity of cellular free cholesterol and cholesterol was determined by thin layer chromatography in order to measure percent oxidation of the cellular free cholesterol. Radioactivity of cellular free cholesterol was 2655 ± 550 cpm/dish, and the ratio of intracellular free cholesterol to total cellular cholesterol was 52.4 ± 3.4% in radioactivity. Cellular protein was 342.4 ± 12.4 μg/dish.

**Table IV**

|                  | Control | Activated | Sphingosine |
|------------------|---------|-----------|-------------|
| ApoA-I           | 2.24 ± 0.25 | 3.25 ± 0.24* | 2.42 ± 0.05 |
| Emulsion         | 6.32 ± 0.43 | 5.83 ± 0.30 | 6.53 ± 0.48 |

* p < 0.01.
achieved, but marginally by the pretreatment with PDGF alone and more prominently when the cells were pretreated with PDGF and MCSF. Without the stimulation, cholesterol was poorly incorporated into the apoA-I-mediated lipid efflux, generating cholesterol-poor pre-β-HDL in the medium. Stimulation of the cells by PDGF and MCSF plus PMA enhanced the apoA-I-mediated cholesterol efflux without changing phospholipid efflux rate, generating pre-β-HDL more rich in cholesterol. Interestingly, no effect was found on a nonspecific physicochemical lipid efflux pathway to lipid microemulsions by the same treatment of the cells. The effect of the treatment on cholesterol efflux to HDL was only partial. The observation was consistent with the view that the stimulation is specific to cholesterol incorporation to the apoA-I-mediated cellular lipid efflux, while the interaction of apoA-I with the cell itself is unaffected, since phosphatidylcholine efflux was not influenced by these treatment and the same amount of pre-β-HDL seemed to be produced regardless of the treatment. The partial effect on the HDL-mediated cholesterol efflux may reflect contribution of the apoA-I-mediated mechanism to the overall cellular lipid efflux to HDL.

At least three protein kinase C inhibitors reversed the stimulating effect of PMA on the apoA-I-mediated cholesterol efflux from the vascular smooth muscle cells pretreated with PDGF and MCSF, again without changing the cellular phospholipid efflux by free apoA-I. The inhibitors had no effect on the nonspecific cellular lipid efflux to lipid microemulsions. In contrast, intracellular cholesterol esterification was suppressed by the same PMA treatment in the pretreated vascular smooth muscle cells with PDGF and MCSF, and this effect was also reversed by the protein kinase C inhibitor. However, overall cellular free cholesterol pool size was unaffected by these cellular treatment and probing of cellular surface cholesterol by extracellular cholesterol oxidase did not demonstrate any significant difference between the control cells and the stimulated cells. The data thus indicated that intracellular cholesterol is translocated to the specific pool readily available to apoA-I-mediated lipid efflux by the short term incubation of the pretreated cells with PMA, and this was inhibited by the protein kinase C inhibitors. This effect was, however, not to an extent that influences cellular surface lipid composition reflecting in nonspecific physicochemical cellular lipid efflux and probing surface cholesterol by cholesterol oxidase.

In the previous few years, a concept of a specific and biologically regulated cellular cholesterol efflux has been gradually developed by several research groups including ourselves. The efflux is mediated by lipid-free helical apolipoproteins and the reaction results in generation of pre-β-HDL with the apolipoprotein and cellular phospholipid and cholesterol (Hara and Yokoyama, 1991, 1992; Hara et al., 1992; Bielicki et al., 1992; Forte et al., 1993). This efflux reaction is distinguishable from nonspecific physicochemical lipid exchange between cell and lipoprotein in many aspects. The reaction has substantially lower $K_m$ (in the order of $10^{-7} \text{M}$) (Hara and Yokoyama, 1991;
number of macrophage-like functions, including expression of Thp-1 cells with respect to a
1994), and Pma is used for inducing differentiation of macro-
ceptor and the MCSF receptor in certain conditions such as stimulation by PDGF BB (Naba et al., 1992a, 1992b). MCSF is known to enhance the activity of macrophage (Nakoinz and Ralph, 1988; Hume et al., 1988; Shibashi et al., 1990; Shimano et al., 1990; Mori et al., 1991; Naba et al., 1993; Ishii et al., 1994), and PMA is used for inducing differentiation of macro-
either to HDL or by lipid-free apoA-I. When the two growth factors were used in combinations, additional PMA caused prominent specific stimulation of cholesterol availability for lipid-free apoA-I-mediated lipid efflux while phospholipid efflux in this pathway was unchanged. Accordingly, this resulted in generating pre-β-HDL particles with higher cholesterol/ phospholipid ratios than the particles generated with the non-
Stimulation of the smooth muscle cells by these factors
not be distinguished from the cellular cholesterol pool that under-
interpret these results, and the regulation would be strictly lim-
the apolipoprotein-mediated efflux may have to be introduced to
mesenchyme. However, the attempt of probing surface cholesterol by extracel-
the apolipoprotein-mediated lipid efflux (Li et al., 1995). Specific defect of this pathway has been identified in the fibroblasts from the pa-
ting to the specific pool readily available for the apolipoprotein-
mediated efflux is induced by PMA and prevented by the
LDL, and HDL (100 μg of protein). The values are percentage of cellular free cholesterol pool representing mean ± S.E. of four data.

K_m, 1993; Li et al., 1993). Extracellular cholesterol esteri-
mediated lipid efflux (Li et al., 1995). The two distinct

The stimulation of cholesterol efflux was independent of

**Table V**
The effect of PDGF, MCSF, and PMA on the lipid efflux from macrophages
The cholesterol-loaded macrophages (75.2 ± 7.2 μg of protein/dish, free cholesterol was 58.1 ± 1.8% of total cellular cholesterol (7.8 ± 1.7 μg)) were pretreated with PDGF (10 ng) and/or MCSF (100 ng) and then stimulated by PMA (160 nM) as described in the text. The efflux of cholesterol was observed for 24 h, in the presence of apoA-I (10 μg) and HDL (100 μg of protein). The values are percentage of cellular free cholesterol pool represented mean ± S.E. of four data.

|          | Control | PMA  | PMA + PDGF | PMA + MCSF | PMA + PDGF/MCSF |
|----------|---------|------|------------|------------|-----------------|
| ApoA-I   | 22.6 ± 6.4 | 29.5 ± 4.9 | 32.9 ± 3.3 | 32.1 ± 6.5 | 27.6 ± 8.4 |
| HDL      | 98.0 ± 6.1 | 79.1 ± 13.2 | 85.8 ± 24.7 | 66.6 ± 13.3 | 90.3 ± 22.5 |

Regulation of Apolipoprotein-mediated Cholesterol Efflux

The stimulation of mouse macrophages by human PDGF, MCSF, and PMA, or combinations of these factors, did not demonstrate
Regulation of Apolipoprotein-mediated Cholesterol Efflux

It is increasingly important to study the cellular factors in regulation of cholesterol efflux by the lipid-free apolipoprotein-mediated pathway. It seems to be particularly important to investigate its regulation by intracellular trafficking of cholesterol in relation to such a factor as manipulation of cellular protein phosphorylation in order to understand the entire process. Since apolipoprotein-cell interaction seems to be a major source of human plasma HDL (Francis et al., 1995), it is increasingly important to study the cellular factors involved in this reaction system.

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